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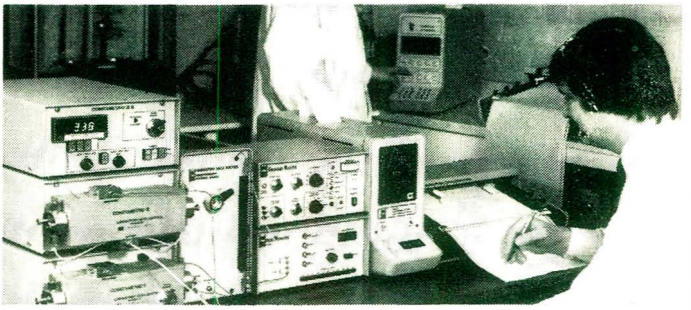

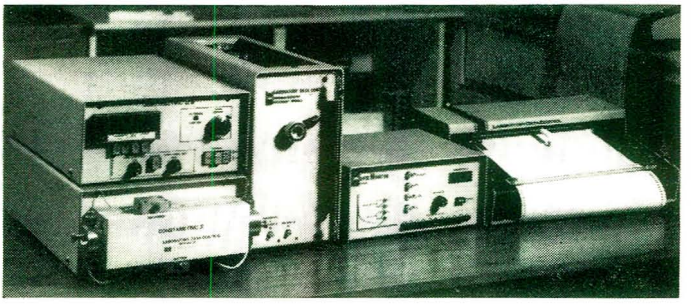

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QUANTITATIVE ANALYSIS OF STEROID PROFILES FROM URINE BY CAPILLARY GAS CHROMATOGRAPHY

I. ACCURACY AND REPRODUCIBILITY OF THE SAMPLE PREPARATION

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(First received February 14th, 1978; revised manuscript received May 6th, 1978)

SUMMARY

A method is described for the determination of steroid profiles from urine by means of gas chromatography using high-efficiency glass capillary columns. The accuracy and reproducibility of essential steps in the sample preparation (extraction of steroids and steroid conjugates by means of XAD-2, enzymatic hydrolysis with *Helix pomatia* juice, solvolysis in acidified ethyl acetate and alkali wash) are established using different endogenously labelled urine samples, obtained from normal subjects to whom labelled steroids had been administered. Preliminary results are given on the reproducibility of the derivatization procedure (formation of methoxime-trimethylsilyl (MO-TMS) ethers), the gas chromatographic analysis and the whole method. Two procedures for the purification of MO-TMS steroid derivatives are compared. Application of the method to urine samples of patients with various endocrine disorders is included.

INTRODUCTION

Many papers have been published in recent years on different aspects of the determination of steroid profiles from urine by gas chromatography (GC).

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Identification number of peaks in the figures	Trivial name	Abbreviation	Systematic name
1	Androsterone	A	3α-Hydroxy-5α-androstane-17-one
2	Etiocholanolone	E	3α-Hydroxy-5β-androstane-17-one
3	Dehydroepiandrosterone	DHEA	3β-Hydroxy-5-androstene-17-one
4	11-Keto-androsterone	11-O-A	3α-Hydroxy-5α-androstane-11,17-dione
	11-Keto-etiocholanolone	11-O-E	3α-Hydroxy-5β-androstane-11,17-dione
5	Estradiol	E-II	3,17β-Dihydroxy-1,3,5(10)-estratriene
6	11β-Hydroxy-androsterone	11-OH-A	3α,11β-Dihydroxy-5α-androstane-17-one
7	11β-Hydroxy-etiocholanolone	11-OH-E	3α,11β-Dihydroxy-5β-androstane-17-one
8	Pregnanediol	PD	3α,20α-Dihydroxy-5β-pregnane
9	Allopregnanediol	aPD	3α,20α-Dihydroxy-5α-pregnane
10	Pregnanetriol	PT	3α,17α,20α-Trihydroxy-5β-pregnane
11	THS		3α,17α-21-Trihydroxy-5β-pregnane-20-one
12	THDOC		3α,21-Dihydroxy-5β-pregnane-20-one
13	Estriol	E-III	3,16α,17β-Trihydroxy-1,3,5(10)-estratriene
14	aTHS		3α,17α,21-Trihydroxy-5α-pregnane-20-one
	aTHDOC		3α,21-Dihydroxy-5α-pregnane-20-one
15	THE		3α,17α,21-Trihydroxy-5β-pregnane-11,20-dione
16	THA		3α,21-Dihydroxy-5β-pregnane-11,20-dione
17	THB		3α,11β,21-Trihydroxy-5β-pregnane-20-one
18	aTHB		3α,11β,21-Trihydroxy-5α-pregnane-20-one
19	THF		3α,11β,17α,21-Tetrahydroxy-5β-pregnane-20-one
	aTHE		3α,17α,21-Trihydroxy-5α-pregnane-11,20-dione
20	aTHF		3α,11β,17α,21-Tetrahydroxy-5α-pregnane-20-one
21	α-Cortolone		3α,17α,20α,21-Tetrahydroxy-5β-pregnane-11-one
22	β-Cortolone		3α,17α,20β,21-Tetrahydroxy-5β-pregnane-11-one
	β-Cortol		3α,11β,17α,20β,21-Pentahydroxy-5β-pregnane
23	α-Cortol		3α,11β,17α,20α-21-Pentahydroxy-5β-pregnane
	Estrone	E-I	3-Hydroxy-1,3,5(10)-estratriene-17-one
	Cortisone		17α,21-Dihydroxy-4-pregnene-3,11,20-trione
	Androstenedione		4-Androstene-3,17-dione
	Testosterone	T	17β-Hydroxy-4-androstene-3-one

Most of them deal with the analytical procedure, for example, the development of techniques for sample preparation [1—8], the formation of suitable derivatives [9—14], or the preparation of glass capillary columns [15—20]. Other papers [21—35] describe applications of this method to problems of clinical interest, showing the great possibilities of the techniques employed. It is likely that in the future steroid profiling by means of GC using high-efficiency glass capillary columns will be used more often, since no other technique gives qualitative and quantitative information on each steroid present in the sample from only one determination.

In our opinion, until now not enough attention has been paid to quantitative aspects of the analysis, especially sample preparation i.e. extraction of steroids and steroid conjugates from urine, hydrolysis, solvolysis and alkali wash. It is realized that when large deviations from the normal pattern have to be established, no quantitative method is required. However, small deviations can only be detected with reliable quantitative methods. Some clinical problems underline the need for such a method.

In some papers methods for sample preparation were tested with only a few selected steroids [3, 23—26], or steroid conjugates [6, 23—28], and the results generalized to all classes of steroids and all types of steroid conjugates. In a series of papers a method for the quantitative GC analysis of steroid profiles from urine will be described. This method will be evaluated for all classes of steroids and all types of steroid conjugates normally present in urine samples. In this first paper we describe the quantitative evaluation of essential steps in the preparation of the sample. Preliminary results on derivatisation and GC analysis are given.

Sample preparation procedures can only be evaluated correctly if urine samples are used in which "endogenously" labelled conjugates are present, the samples being obtained from normal subjects to whom labelled steroids had been administered orally or by continuous infusion. As these labelled steroids take part in normal metabolism, all metabolites of these compounds will appear in the urine as labelled conjugates. To our knowledge such investigations have been described only by Bradlow [1] and Setchell et al. [2] for a small number of steroids. In this study normal subjects were given cortisol*, androstenediol and estrone, androstenedione and DHEA, or DHEA—S and DHEA. The main metabolites of cortisol [36] are THE, aTHE, THF, aTHF, α- and β-cortolones and cortols, 11-O-A and 11-O-E, 11-OH-A and 11-OH-E. Androsterone, etiocholanolone and testosterone are the most important metabolites of androstenedione. Estrone is partly converted into estriol, estradiol and other estrogens. Infusion with DHEA—S results in the excretion of DHEA, androsterone and etiocholanolone. Using urine samples obtained from normal subjects to whom the above-mentioned steroids had been administered, the whole polarity range of steroids and all types of steroid conjugates, normally present in urine samples, are included.

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*Trivial names and abbreviations used in this paper are listed in Table I. A capital G or S added to the steroid name indicates the glucuronide or sulphate conjugate, respectively.

EXPERIMENTAL

Materials

Non-radioactive steroids were purchased from Steraloids (Pawling, N.Y., U.S.A.) and Ikapharm (Ramat Gan, Israel). Radioactive steroids, [4-^{14}C]-androstenedione (60 mCi/mmole), [4-^{14}C]dehydroepiandrosterone sulphate, ammonium salt (54 mCi/mmole), [1,2-^{3}H]cortisol (0.36 Ci/mmole), [6,7-^{3}H]-estrone (44 Ci/mmole) and [7-^{3}H]dehydroepiandrosterone (16.6 Ci/mmole), were all purchased from the Radiochemical Centre (Amersham, Great Britain) and used only after a chromatographic purity check. Servachrom XAD-2 (Serva, Heidelberg, G.F.R., particle size 300—1000 μm) was purified according to the method described by Setchell et al. [12]. In some experiments Amberlite XAD-2 (Rohm & Haas, Pa., U.S.A., particle size 300—1000 μm) was used after purification according to the method of Shackleton et al. [6]. *Helix pomatia* juice was obtained from Industrie Biologique Française (Gennevilliers, France). Lipidex-5000, obtained from Packard-Becker (Groningen, The Netherlands), was purified according to the method of Axelson et al. [37]. Solvents (ethanol, ethyl acetate, hexane) were p.a.-grade (Merck, Darmstadt, G.F.R.) and used without further purification. Pyridine (Merck) was redistilled before use over P_2O_5 and stored over potassium hydroxide. Methoxyamine hydrochloride was purchased from Applied Science Labs., (State College, Pa., U.S.A.). Hexamethyldimethylsilazane (HMDS) was obtained from Pierce (Rockford, Ill., U.S.A.). Dimethoxypropane was purchased from Aldrich (Beerse, Belgium). The silylation mixture, BSA·(N,O-bistrimethylsilylacetamide), TSIM (N-trimethylsilylimidazole) and TMCS (trimethylchlorosilane) in the ratio 3:3:2 (v/v) was purchased from Supelco (Bellefonte, Pa., U.S.A.). Scintillation cocktail NE 262 was supplied by Nuclear Enterprises (Edinburgh, Great Britain). Urine samples were obtained from normal subjects to whom had been administered ^{3}H-labelled cortisol (1 μCi, oral), ^{3}H-labelled estrone/^{14}C-labelled androstenedione, ^{3}H-labelled DHEA/^{14}C-labelled DHEA—S or ^{3}H-labelled DHEA/^{14}C-labelled androstenedione (30 μCi ^{3}H and 15 μCi ^{14}C, all given by continuous infusion for, respectively, 2 and 12 h).

Sample preparation

Extraction of steroids and steroid conjugates from urine. Urine samples (24 h) were collected in polyethylene bottles. After the volume had been recorded, they were stored at —20° before processing. Urine (10 ml) was passed through a column (10 × 1 cm I.D.) containing 8 g of XAD-2 resin. The column was fitted with a 25-ml reservoir and a PTFE stopcock. After being washed with 20 ml of distilled water, the column was allowed to drain completely. Steroid conjugates were eluted with 40 ml of methanol. The flow-rate was kept constant (0.5—1.0 ml/min) throughout the entire procedure. Columns can be re-used after washing three times with 40 ml of distilled water and removing air bubbles.

Hydrolysis and solvolysis. Methanol was evaporated under nitrogen at 60°. The residue was redissolved in 10 ml of sodium acetate buffer (0.5 *M*, pH 5.0), to which 0.1 ml of *Helix pomatia* juice was added containing 10,000

Fishman units of β-glucuronidase and 80,000 Roy units of sulphatase. Hydrolysis of the steroid glucuronides was performed for 18 h at 37°. After hydrolysis the sample was brought to pH 1 with concentrated hydrochloric acid, and NaCl (1.5 g) was added [38]. Liberated steroids and steroid sulphates were extracted by shaking with 25 ml of ethyl acetate for 60 min. The aqueous phase was removed by suction and solvolysis took place for 18 h at 45°. Only when the accuracy of the hydrolysis and solvolysis had to be determined was ethyl acetate evaporated under nitrogen at 60°. The residue was redissolved in 10 ml of distilled water and liberated steroids were extracted three times each with 10 ml of ethyl acetate.

Alkali wash. The ethyl acetate fraction was washed twice with 5 ml of 8% aqueous sodium bicarbonate (to remove acids but not phenols) and two times with 5 ml of distilled water. It was shown that some steroids were partly lost in the aqueous phase. These steroids could be recovered by XAD-2 extraction as already described. Finally, the combined organic layers were evaporated under a stream of nitrogen at 60°.

Formation of derivatives

Preparation of methoxime-trimethylsilyl (MO-TMS) derivatives. The dry residue was redissolved in 1 ml of ethyl acetate, transferred to a silylation vial and 1 ml of a solution of *n*-alkanes C_{24} and C_{32} in hexane (3 mg/100 ml) was added. Solvents were evaporated under a stream of nitrogen at 60°. To the dry residue 100 µl of a solution of methoxyamine hydrochloride (100 mg/ml) in dry pyridine were added. The vial was closed with a PTFE-lined cap and heated for 1 h at 60°. Excess pyridine was removed under nitrogen at 60°. After the addition of 100 µl of the mixture BSA—TSIM—TMCS (3:3:2, v/v), persilylation was achieved in 4 h at 80° for all compounds, except for cortols and cortolones; full silylation of these latter compounds took 24 h at 80°.

Purification of derivatives. Two methods were employed for purification of the sample after derivatisation.

Method A. Excess silylation reagents and compounds with polarities similar to those of steroids, were removed on a Lipidex-5000 column (70 × 4 mm) containing 0.25 g of dry gel, prepared in the solvent system hexane—HMDS—pyridine—dimethoxypropane (97:1:2:10, v/v) [2, 37, 39]. The sample was transferred onto the top of the column by adding 400 µl of the solvent to the reaction mixture. The vial was washed with 500 µl of the solvent, which was also passed through the column. For rapid filtration a nitrogen pressure of 0.5 kg/cm^2 was applied, resulting in a flow of 3 ml/min. MO-TMS derivatives were recovered in the first 3.5 ml of effluent. Solvents were evaporated under nitrogen at 60°. The sample was redissolved in 1 ml of hexane, of which 1 µl was injected into the gas chromatograph.

Method B [40]. To the reaction mixture 1 ml of methylene chloride was added. Excess reagents were removed by washing the methylene chloride with 1 ml of 0.1 *N* sulphuric acid and twice with 1 ml of distilled water. The sample was dried over anhydrous sodium sulphate and 1 µl of the supernatant was injected into the gas chromatograph. A flow diagram of the complete method is shown in Fig. 1.

Liquid scintillation counting. The accuracy and reproducibility of the

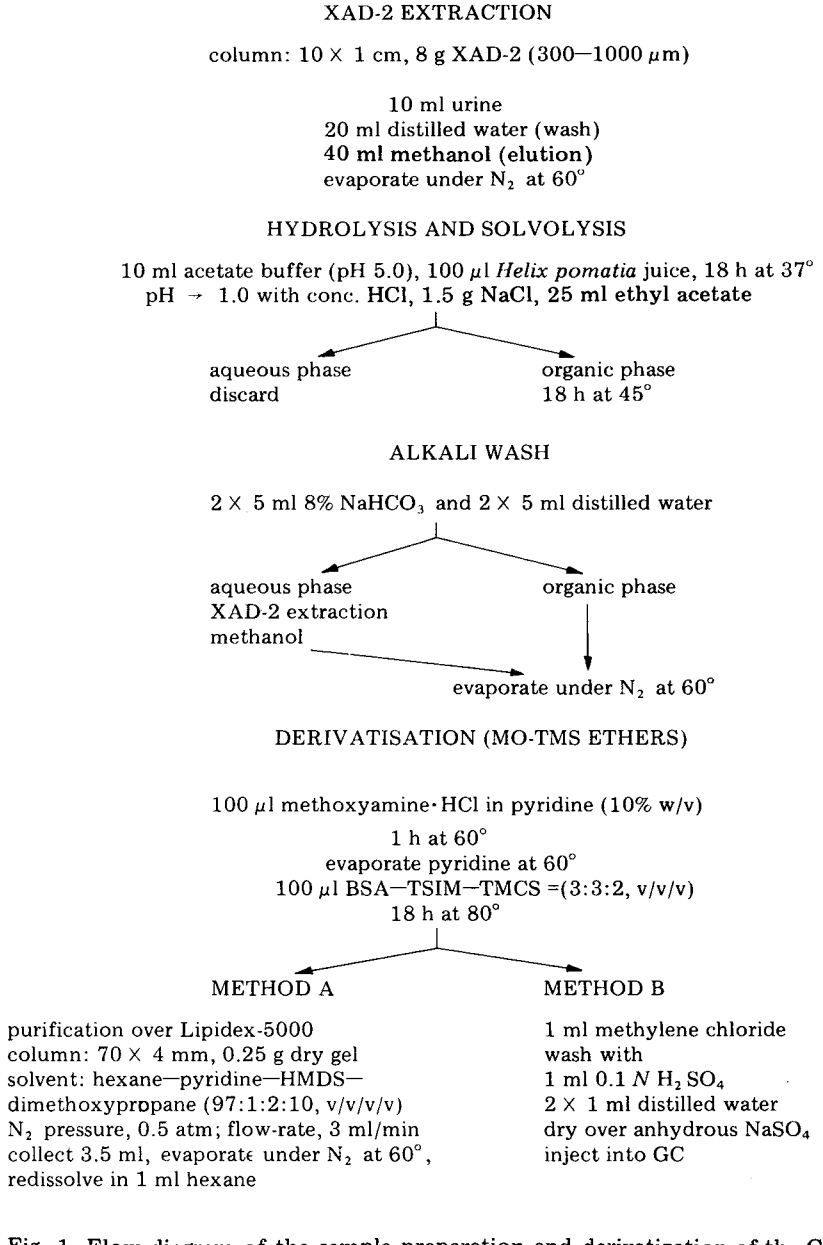

Fig. 1. Flow diagram of the sample preparation and derivatization of the GC determination of urinary steroid profiles.

sample preparation procedure was established by counting 1-ml samples in 11 ml of the scintillation cocktail. Liquid scintillation counting was performed in a Packard TriCarb 2003 (Packard, Brussels, Belgium), or a Mark III (Searle Analytic, Des Plaines, Ill., U.S.A.) equipped with automatic quench correction, for 10 min or until 10,000 disintegrations had been counted.

Gas chromatography

Preparation of columns. Glass capillary columns were prepared according to the method described by Rutten and Luyten [20]. Columns (0.25 mm I.D. and 1.0 mm O.D.) were drawn from Pyrex glass tubes. The internal diameter was carefully kept constant during the drawing process. Columns were de-activated with benzyltriphenylphosphonium chloride (BTPPC; Aldrich) and coated with a solution of 0.25% (w/w) SE-30 (Merck) in hexane using the static procedure [41].

Instrumentation. GC was carried out using a Perkin-Elmer F 30 gas chromatograph, equipped with an all-glass solid injector [42] and modified for the use of glass capillary columns. A home-made gas chromatograph was also used, constructed with a Becker 1452 D air thermostat and Becker gas-flow regulators. Carrier gas (nitrogen) was controlled by a precision pressure controller (Wallace and Tiernan, Gunzburg, G.F.R.). The column was housed in an aluminium block to minimize the effect of temperature fluctuations in the oven. Samples were injected with a moving-needle injection system [42]. The flame ionisation detector and the amplifier were also home-built.

Identification of steroids

Kováts indices [43] were measured for 27 reference steroids on SE-30 at 250°. The inlet pressure of the carrier gas (nitrogen) was 1.0 kg/cm^2. The logarithmic plot of even-numbered *n*-alkanes between C_{24} and C_{32} was found to be linear. Indices were calculated by linear interpolation between the outer two *n*-alkanes. Times were measured by a chronograph and the appearance of the solvent was taken as inert gas time. Steroids in urine samples were identified by measuring Kováts indices under the same conditions as the reference steroids. When identification was only tentatively possible, because of small differences in the Kováts index, gas chromatography—mass spectrometry (GC—MS) was used to confirm the identity of the compound involved. Mass spectra of the 27 reference steroids as their MO-TMS derivatives were recorded by GC—MS using an AEI MS-12 mass spectrometer (trap-current 500 μA, source temperature 255°, accelerating voltage 4 kV, electron energy 70 eV, magnetic scan speed 2 sec/decade). GC was performed at 250° on SE-30. The gas chromatograph was directly coupled to the mass spectrometer [44]. No attempts were made in this study to establish the identity of minor compounds in the profile.

RESULTS AND DISCUSSION

Sample preparation

First, the accuracy and reproducibility of the XAD-2 extraction, hydrolysis/solvolysis and alkali wash were established in separate experiments. Subsequently, the overall recovery of the whole sample preparation was determined by carrying out the extraction, hydrolysis/solvolysis and alkali wash without establishing the recoveries of the individual steps. In this way calculated overall recoveries can be compared with values measured in separate experiments. The results are given in Table II.

TABLE II

ACCURACY AND REPRODUCIBILITY OF SAMPLE PREPARATION

Values are expressed as percentages ± standard deviation. NM: not measured.

	Steroid(s) administered						
	^{3}H-Labelled cortisol*	^{14}C-Labelled androstenedione** + ^{3}H-labelled DHEA		^{14}C-Labelled androstenedione** + ^{3}H-labelled estrone		^{14}C-Labelled DHEA—S** + ^{3}H-labelled DHEA	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
XAD-2 Recovery	94 ± 5 (n = 73)	100 ± 3	94 ± 4	103 ± 3	94 ± 5	100 ± 1	96 ± 3
Hydrolysis and solvolysis	95 ± 8 (n = 43)	93 ± 4	87 ± 2	96 ± 3	91 ± 1	86 ± 3	83 ± 4
Alkali wash	NM	99 ± 4	97 ± 5	96 ± 3	84 ± 9	101 ± 3	101 ± 3
Overall recovery: calculated	89	92	79	95	72	87	80
Overall recovery: measured in separate experiments	88 ± 3 (n = 16)	92 ± 3	80 ± 5	96 ± 2	71 ± 7	87 ± 3	81 ± 2

*Seven different urine samples, including 13 series.
**Number of experiments for dual-labelled samples = 8.

XAD-2 procedure

Most of the experiments were carried out with urine samples of subjects to whom ^{3}H-labelled cortisol had been administered. Samples of seven different subjects were used; 13 series were carried out in two different laboratories by two technicians. No differences could be detected between the results obtained from either different urine samples or in different laboratories. For the extraction of steroid conjugates and steroids from urine, the batches of XAD-2 obtained from Serva and Rohm & Haas, gave equally good results.

We preferred to purify the resin using the method of Setchell et al. [2], since purification according to the method of Shackleton et al. [6] does not remove all impurities detectable by GC. Elution of steroids and steroid conjugates with 50 ml of methanol instead of 40 ml did not increase the recovery. The XAD-2 extraction resulted in a mean recovery of 94.5 ± 5.5% (n = 73). When 15 ml of urine instead of 10 ml were passed through the XAD-2 columns, significantly lower recoveries were found (87.5%, n = 20), while the standard deviation increased to 9.6%. Experiments with 10 ml of a urine sample from a patient suffering from Cushing's syndrome and excreting about ten times as much cortisol metabolites as normal subjects, yielded a XAD-2 recovery of 84.8 ± 3.4% (n = 8), 5% of the radioactivity being lost in the aqueous effluent. Only after a second elution with 15 ml of methanol could the remaining 10% of radioactivity be recovered from the columns. Care must therefore be taken to avoid overloading the column when urine samples of patients excreting abnormally large quantities of steroid conjugates are carried through the procedure. To avoid contamination of further experiments with the same column, we suggest that the columns are regenerated not only by washing with water, but also with 40 ml of methanol.

For the metabolites of androstenedione, estrone, DHEA and DHEA—S also, an almost quantitative recovery from the XAD-2 columns was obtained. Good agreement was found for urine samples from different normal subjects to whom identical steroids (androstenedione or DHEA) had been administered (Table II). The results, given above, agree well with those obtained by other workers [1, 2, 8, 23—26, 45].

Hydrolysis and solvolysis

For the quantitative analysis of steroid profiles, enzymatic hydrolysis must be followed by solvolysis [2,6,7,23,24], since the sulphatases present in *Helix pomatia* juice are not able to hydrolyse sulphate conjugates of a 3α-OH group in 5α-steroids [52], and of 17- and 20- hydroxyl groups [refs. 53 and 6, respectively]. In human urines these conjugates were found to be excreted in varying amounts. For cortisol metabolites the mean recovery of hydrolysis plus solvolysis was 95.0 ± 8.3% (n = 44). The high standard deviation is probably caused by small differences in enzyme activity, since several batches were used throughout this study. Hydrolysis and solvolysis temperatures could be varied between 35 and 50°, and 45 and 50°, respectively, without a significant change in the results. Maximum cleavage of steroid conjugates was achieved within 18 h for both hydrolysis and solvolysis.

These results also prove that of the quantitatively most important corticosteroid metabolites only a minor part is lost during the solvolysis procedure. It must be emphasized that solvolysis is not suitable for the analysis of corticosteroids for which artefact formation is known to occur, such as highly polar corticosteroids, normally present in almost negligible amounts in urine.

Hydrolysis and solvolysis of the metabolites of estrone, androstenedione, DHEA and DHEA—S also yielded satisfactory results. They are of the same order of magnitude as those reported by others [1, 3, 6, 7, 23—28, 45]. About 10% of the metabolites of DHEA and DHEA—S were lost with the aqueous phase after hydrolysis. Further experiments need to be carried out to find out whether these metabolites are not hydrolysed or are not extracted by ethyl acetate.

Alkali wash

Further purification of the sample (removal of acids) is achieved by washing the ethyl acetate fraction after solvolysis with aqueous sodium bicarbonate and water. Some highly polar steroids are not completely recovered or are altered during the alkali wash [6].

Alternative procedures using the anion exchanger Amberlyst A-26 suffer from the disadvantage that the recovery of some steroids is not quantitative. The use of diethylaminohydroxypropyl Sephadex LH-20 (DEAP-LH-20) seems to overcome these problems [39]. Since DEAP-LH-20 is not yet commercially available, we have used the alkali wash for purifying samples. Table II shows that almost no steroids are lost if this method of purification is used, with the exception of estrogens. About 15% of the metabolites of estrone are lost on the XAD-2 columns when the combined aqueous phases are passed through the columns and during the wash with distilled water. The reason for

this phenomenon is not yet fully understood, and further experiments will have to be carried out.

Overall recovery

From the data obtained for the individual steps, overall recoveries were calculated by multiplication. In separate experiments, the same urines were taken through the complete procedure without establishing the recoveries of the individual steps; only the resulting overall recovery was measured. From the results, presented in Table II, the excellent agreement between the calculated and measured recoveries is evident.

Preparation and purification of MO-TMS derivatives

MO-TMS derivatives were prepared according to the method described by Horning and co-workers [9—11, 13, 14] with small modifications. For persilylation the mixture BSA—TSIM—TMCS (3:3:2; v/v) [9] was used at 80° to shorten the reaction time. Optimum yield of derivatives was achieved in 4 h, except for cortols and cortolones. Full silylation of these substances required 24 h at 80°. No attempts were made to establish the accuracy of the derivatization procedure because of the absence of flame factors. The reproducibility of the method is given below. Direct injection of the reaction mixture using the moving-needle solid-injection system [42] is impossible owing to the low volatility of the silylating reagents, especially TSIM. Samples must therefore be purified before injection. For this reason the two methods described above were compared. Figs. 2 and 3 show urinary steroid profiles of a patient with Cushing's syndrome, obtained after purification of the MO-TMS derivatives by the two methods. It can be seen that even for minor compounds identical results were obtained. No specific loss of steroids or groups of steroids occurred with either of the methods. Purification of the sample with Lipidex-5000 according to the method described by Axelson and Sjövall [2, 37, 39] yielded a cleaner chromatogram, although differences were small. The method of De Jong [40] is less time-consuming and can

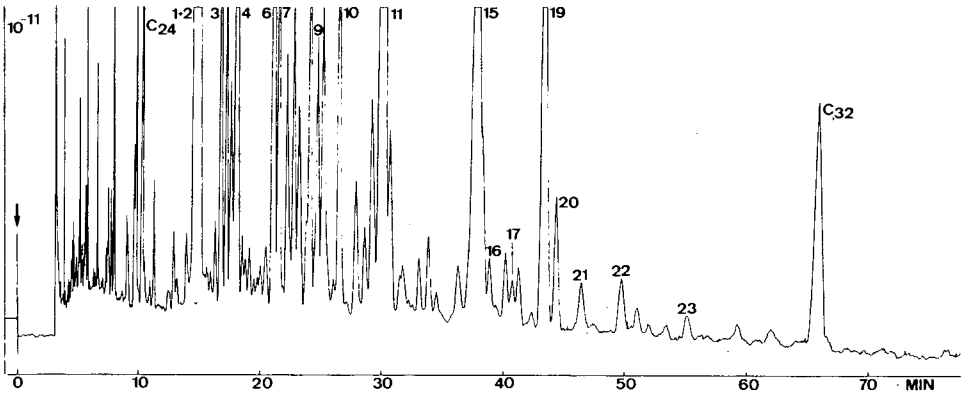

Fig. 2. Steroid profile of a patient with Cushing's syndrome. The sample was purified after derivatization following method A. GC conditions: 35 m × 0.25 mm WCOT SE-30 column; temperature, 250°; pressure of carrier gas (nitrogen), 1.0 kg/cm²; 1.0 μl of the sample was injected. The key to the numbering of the peaks is given in Table I.

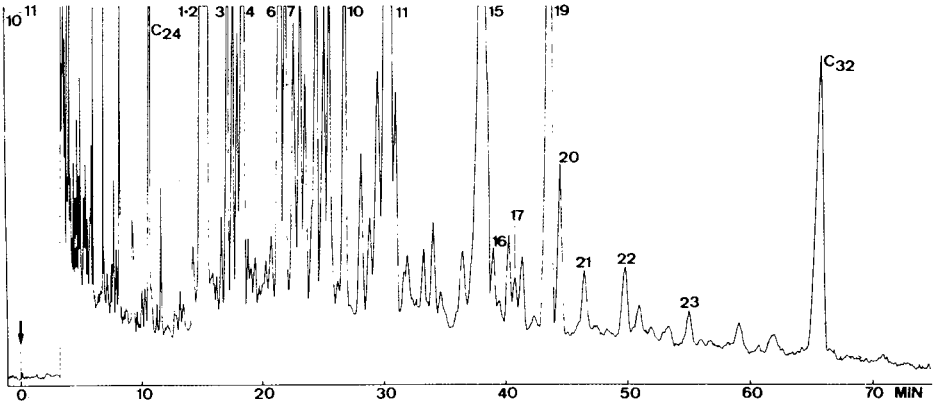

Fig. 3. Profile of the same sample as in Fig. 2, after purification of the MO-TMS derivatives following method B. GC conditions are the same as in Fig. 2.

therefore be recommended for routine analyses. If samples are to be analysed by GC—MS, purification over Lipidex-5000 is to be preferred.

Gas chromatographic analysis of MO-TMS derivatives

In most instances WCOT [20, 48] or SCOT [17—19, 49] columns, coated with the apolar phase SE-30, are used for the separation of steroid MO-TMS derivatives. The separation of 27 references steroids as their MO-TMS derivatives is shown in Fig. 4.

Kováts indices of the MO-TMS derivatives of the 27 references steroids were measured; the results are given in Table III. Using the home-made gas chromatograph, the reproducibility for all steroids was better than 0.5 index units (n = 8).

For quantitative analysis, flame factors or calibration constants must be available. However, they are difficult to establish because they depend not only on the choice of the reference compound(s), but also on the stationary phase [3], the purity of the steroids and reference compound(s), column conditions, variation of detector response and efficiency of the derivatisation [50]. Although studies on this subject are still in progress, some preliminary results can be given (assuming all steroids to be quantitatively derivatised). Most flame factors are in the range 0.8—1.0. A relatively poor response (50—70%) is given by some of the 17-desoxycorticosteroids (THDOC, THA, aTHB, aTHDOC) and also by THE. Also the peak obtained from 11-O-A and 11-O-E is rather small. These observations are in agreement with those reported by others [50, 51].

Reproducibilities (expressed as the coefficient of variation) have been measured for the GC analysis by repeated injection of the derivatized reference mixture. Peak areas were calculated relative to the sum of the areas of two co-injected n-alkanes (n-C_{24} and n-C_{32}) using the program described by Wijtvliet [52]. A DCC D 116 E laboratory computer was used for on-line data acquisition and a Burroughs B 7700 computer for off-line data handling. Values were obtained between 1.8 and 11.5% (except for THA: 18%). The highest values were obtained from the smallest peaks, indicating possible

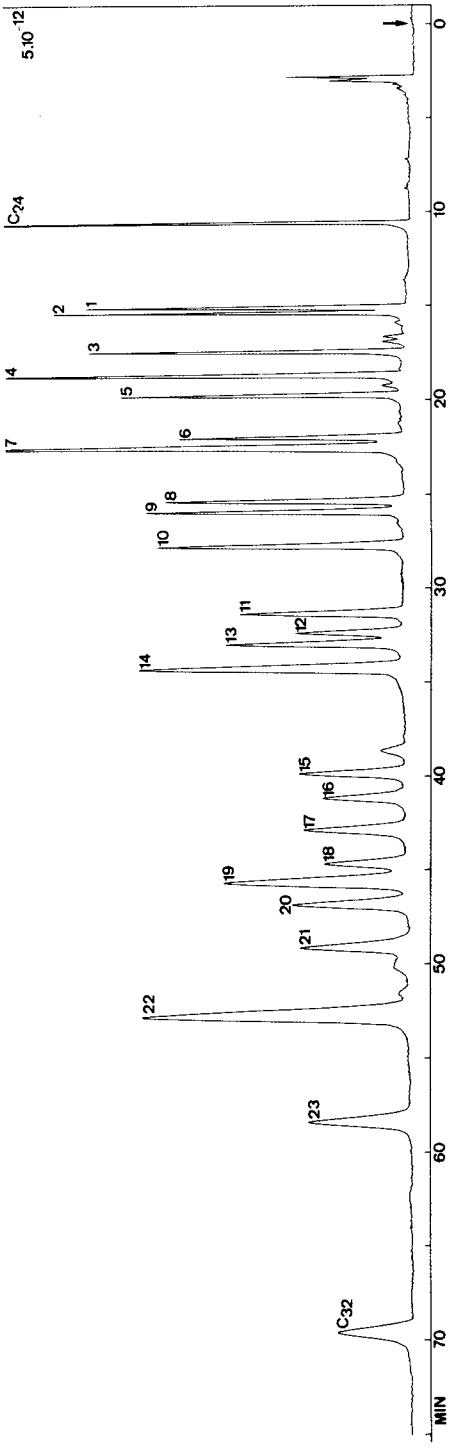

Fig. 4. Separation of the reference mixture on a 35 m × 0.25 mm WCOT SE-30 column. Column temperature, 250°; injection and detection temperatures, 300°; carrier gas (nitrogen) pressure, 1.0 kg/cm^2.

TABLE III

KOVÁTS INDICES OF MO-TMS DERIVATIVES OF STEROIDS

Kováts indices were measured on SE-30 at 250°. Inlet pressure of carrier gas (nitrogen) 1.0 kg/cm².

Peak number	Compound	Kováts index
1	Androsterone	2574
2	Etiocholanolone	2582
3	Dehydroepiandrosterone	2639
4	11-Keto-androsterone	2669
	11-Keto-etiocholanolone	2669
5	Estradiol	2694
6	11β-OH-Androsterone	2738
7	11β-OH-Etiocholanolone	2746
8	Allopregnanediol	2798
9	Pregnanediol	2807
10	Pregnanetriol	2835
11	THS	2884
12	THDOC	2897
13	Estriol	2906
14	aTHS + aTHDOC	2921
15	THE	2981
16	THA	2995
17	THB	3010
18	aTHB	3027
19	THF + aTHE	3036
20	aTHF	3045
21	α-Cortolone	3065
22	β-Cortol	3095
	β-Cortolone	3095
23	α-Cortol	3134

problems with establishing the true baseline. Also a urine sample from a normal male was injected seven times; peak areas ($A = h \times w_{1/2}$) were measured by hand relative to the sum of the peak areas of co-injected n-C_{24} and n-C_{32}. Variation coefficients of between 1.9 and 8.2% were found, the mean variation coefficient being slightly lower than that obtained for the reference mixture.

The reference mixture was derivatized six times in order to establish the reproducibility of the derivatization procedure. Analyses were carried out in the same way as described above. Values obtained ranged from 4.6 to 16.4% (with the exception of THA: 21.3%).

The reproducibility of the whole method, including sample preparation, was established for two different urine samples obtained from a normal adult and a patient with Cushing's syndrome, who was excreting abnormally large quantities of all metabolites. Peak areas were measured by hand. Variation coefficients of the individual steroids varied between 5.8 and 15.8%.

These values agree well with those obtained by others. Shackleton and Honour [50] recently described an automatic solid-injection device able to inject samples with variation coefficients between 1.8 and 8.1%. The re-

producibility of the whole method ranged from 2.4 to 16.6%. Setchell et al. [2], using a more elaborate method, reported variation coefficients of 3—23%. Others obtained variation coefficients of 11—20% [ref. 3] and 7—25% [ref. 22].

Application to human steroid GC profiles

To illustrate the possibilities of the method, GC steroid profiles of a normal male and some patients with various endocrine disorders were determined. Fig. 5 shows a profile of a normal male. Fig. 6 shows the profile of a patient with Cushing's syndrome caused by an adrenal carcinoma. Large quantities

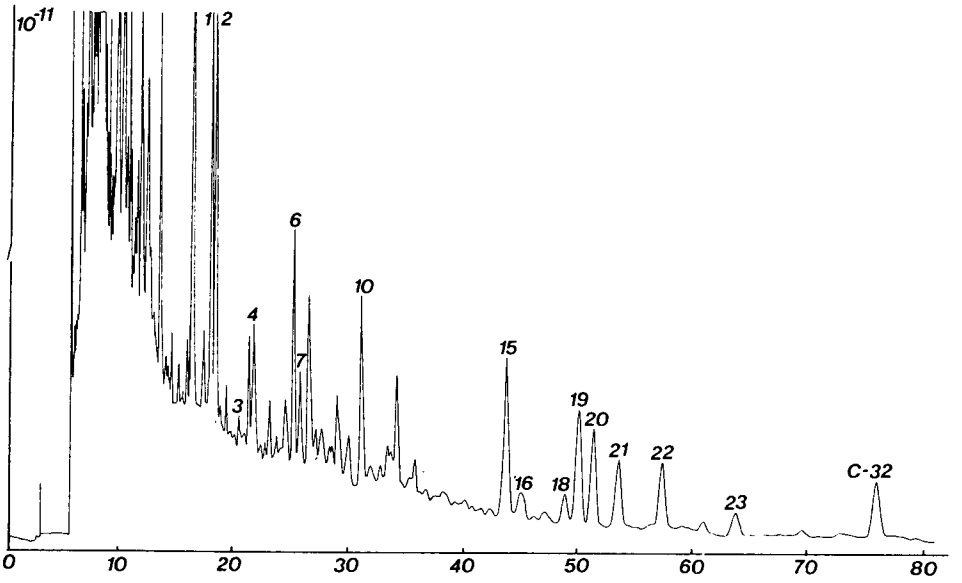

Fig. 5. Steroid profile of a normal male (age 26). GC conditions: 25 m × 0.25 mm WCOT SE-30 column: temperature, 250°; pressure of carrier gas (nitrogen), 1.5 kg/cm^2; 1.0 μl of sample was injected.

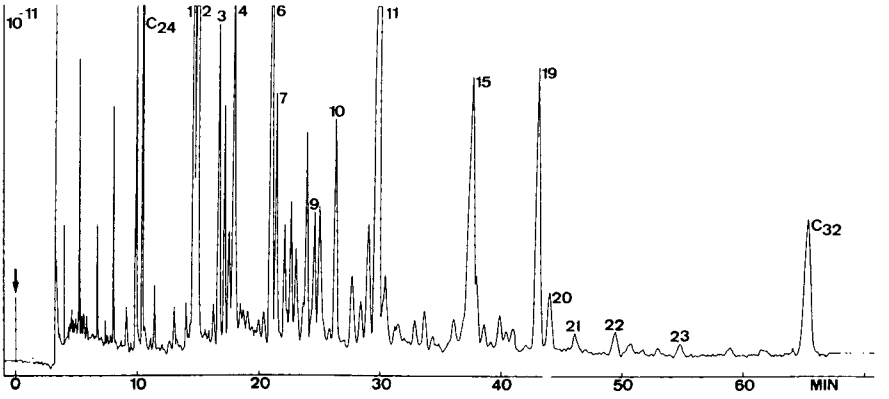

Fig. 6. Steroid profile of a patient with Cushing's syndrome caused by an adrenal carcinoma. GC conditions are the same as in Fig. 2, but only 0.3 μl of sample was injected.

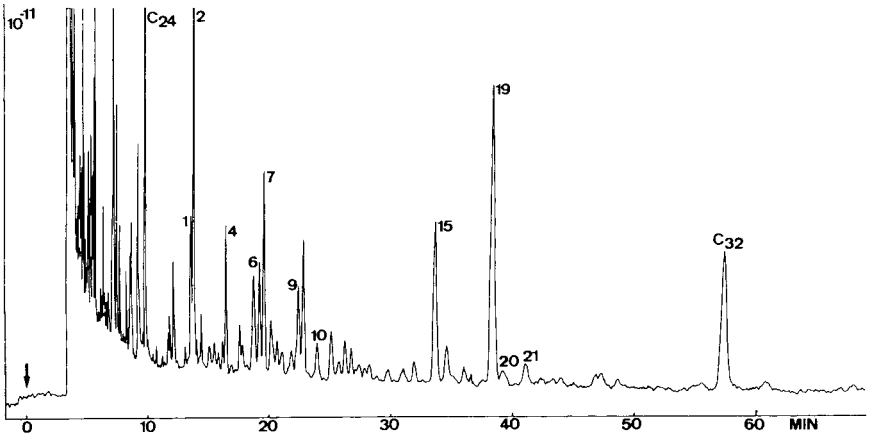

Fig. 7. Steroid profile of a patient with Cushing's syndrome caused by a pituitary tumour. GC conditions are the same as in Fig. 6.

of all steroids are excreted, but the very high excretion of THS is noteworthy. Touchstone et al. [53] and Lipsett et al. [54] had previously reported the production of large amounts of THS by adrenal carcinomas.

The excretion of DHEA is less elevated than is normally found in cases of adrenal carcinoma, although it is known [55] that not all adrenal carcinomas produce large quantities of DHEA. The steroid profile shown in Fig. 7 was obtained from a patient with Cushing's syndrome caused by a pituitary tumour. From this figure some changes in steroid metabolism typical of Cushing's syndrome can be seen. The ratio E/A, normally about 1.0, is increased [53].

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Biomedical Applications

CHROMBIO. 207

PROFILE BEI CHRONISCHEN ERKRANKUNGEN

I. STEROIDPROFILUNTERSUCHUNGEN BEI URAMIE

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SUMMARY

Profiles in chronic diseases. I. Investigations of steroid profiles in uremia

Steroid profiles of hemofiltrates of uremic patients contain as main steroids the sulfates of 11β-hydroxyetiocholanolone, 11-ketoetiocholanolone, 11β-hydroxyandrosterone and 11-ketoandrosterone. In blood of uremic patients androstenediol is the main steroid of the sulfate fraction, while in blood of healthy persons dehydroepiandrosterone sulfate is the main steroid. The gradual decrease of the kidney function is characterized by an increase of 11-oxigenated androstane conjugates in urine.

EINFÜHRUNG

Biologische Flüssigkeiten sind ausserordentlich komplex zusammengesetzt. Überdies kommen die einzelnen Verbindungen in extrem unterschiedlichen Mengenverhältnissen vor, die eine auch nur halbquantitative Erfassung sehr erschweren. Analytische Untersuchungen z.B. mit der Radioimmunoassaymethode konzentrieren sich daher vorzugsweise auf die Erfassung solcher Stoffe, deren Auftreten als Stoffwechselmetabolite bereits bekannt ist. Verbindungen, die unbekannt sind oder als Stoffwechselprodukte nicht erwartet werden, entgehen dem Nachweis.

Die Kombination Glaskapillargaschromatographie—Massenspektrometrie ermöglicht uns heute eine Übersicht über einige Gruppen biologisch interessanter Verbindingen (Steroide, Säuren und Amine) zu erhalten und Abweichungen gegenüber der Norm zu erkennen, falls sich eine Krankheit in einer Änderung des relativen Verhältnisses einzelner Komponenten dieser erfassbaren Substanzklassen niederschlägt.

Diese Analysenmethode scheint uns insbesondere für die Untersuchung chronischer Erkrankungen von Bedeutung zu sein, die häufig mit einer Änderung im Stoffwechselgeschehen verbunden sind. Einige sind vielleicht erst durch eine Stoffwechseländerung bedingt. Mit Sicherheit darf eine Änderung des Stoffwechselgeschehens bei Urämikern angenommen werden, da in diesem Fall bei vollständigem Ausfall der Nieren die schädlichen Stoffe durch Hämodialyse [1, 2] oder nach der neuen Methode der Hämofiltration [3] aus dem Körper entfernt werden müssen.

Es hat nicht an Versuchen gefehlt, durch Untersuchungen der Komponenten des Hämodialysats und des Blutes urämischer Patienten Hinweise auf angehäufte Schadstoffe zu erhalten [4]. Auch die Kombination Gaschromatographie—Massenspektrometrie (GC—MS) wurde zu derartigen Untersuchungen eingesetzt. Jänne et al. [5] fanden mit Hilfe dieser Methode im Blut von Urämikern und Gesunden das 3α-Hydroxy-5-androsten-17-on-sulfat und stellten darüber hinaus fest, dass es im Blut nierenkranker in erhöhter Menge vorhanden war. Von anderen Arbeitsgruppen wurden hauptsächlich Zucker und Säuren untersucht [6, 7].

Die Hauptschwierigkeit, Profile solcher Patienten zu erhalten, lag in der notwendigen Anreicherung der Proben, die in Filtraten und Dialysaten in extrem hoher Verdünnung vorhanden sind: bei der Hämofiltration werden ca. 20 l, bei der Dialyse 120 l Flüssigkeitsvolumen umgesetzt.

Die Erfahrungen, die wir in früheren Arbeiten bei der Untersuchung biologischer Flüssigkeiten gewonnen haben [8, 9], mussten auch eine Analyse von Plasma und Hämofiltratproben urämischer Patienten, besser als es bisher gelang, ermöglichen. Die ersten Ergebnisse erforderten auch die Untersuchung von Urinproben solcher Patienten, die noch fähig waren, Harn auszuscheiden. Über den Gesamtkomplex solcher Untersuchungen soll hier berichtet werden.

PROBENMATERIAL

Insgesamt wurden 14 Hämofiltrate (5 von urämischen Frauen, 7 von urämischen Männern, 2 von nierengesunden Kontrollpersonen), 4 Plasmapools von Urämikern (2 von Männern, 2 von Frauen), 20 Plasmaproben Nierengesunder, 14 Urinproben von Patienten mit eingeschränkten Nierenfunktionen (7 Männern, 7 Frauen) und 20 Urinproben nierengesunder Kontrollpersonen untersucht.

AUFARBEITUNG [8]

Hämofiltrat

Methode A. Jeweils 2 l Hämofiltrat wurde unmittelbar nach der Abnahme über eine XAD-4-Säule gegeben [10] . War eine Aufarbeitung von frischem

Hämofiltrat nicht möglich, so wurde es in der Kühltruhe bei −10° gelagert. Die XAD-Säule (50 × 4 cm I.D., 300 g Füllmaterial) wurde mit 500 ml physiologischer Kochsalzlösung und dann mit 200 ml Wasser gewaschen und der organische Extrakt mit 500 ml Methanol eluiert.

Die Steroide wurden an eine Sephadex-LH-20-Säule [20 × 1 cm, 8 g Füllmaterial; Laufmittelsystem, Methanol—Chloroform (1:1), dem 0.01 Mol NaCl zugesetzt war] in Glucuronide (0—25 ml) und Sulfate (25—100 ml; Laufmittel: Methanol) fraktioniert [11]. Die Konjugatfraktionen wurden mit 25 ml 0.5 *M* Acetatpuffer bei pH 4.7 mit 0.3 ml Helicase enzymatisch verseift [12] und die freigesetzten Steroide nach Horning und Pfaffenberger zuerst zweimal mit jeweils 50 ml Methylenchlorid und dann mit 50 ml Essigester extrahiert [13] . (1 ml Helicase enthält 100,000 Einheiten β-Glucuronidase und 50,000 Einheiten Arylsulfatase).

Anschliessend wurden die Säuren an DEAP-LH-20 abgetrennt [20 × 1 cm, 10 g Füllmaterial; Laufmittel, Methanol—Wasser—Chloroform (9:2:1), 17—40 ml] [14].

Zur Entfernung unpolarer Verbindungen wurde die Probe an Kieselgel chromatographisch gereinigt [200 mg Füllmaterial in Benzol aufgeschlämmt; Laufmittel (a) Benzol—Essigester (95:5); (b) Essigester], wobei unpolare Verbindungen zuerst mit 30 ml Benzol—Essigester entfernt und die Steroide dann mit 30 ml Essigester eluiert wurden [15].

Ein Teil des organischen Extraktes, in Abhängigkeit vom eingesetzten Ausgangsvolumen und Einstellung der Geräte, wurde in einem Schmelzpunktröhrchen mit N-Trimethylsilyl-N-methyl-trifluoracetamid versetzt und unter Luftabschluss 24 h bei Raumtemperatur belassen. Die entstandenen Trimethylsilylether der Steroide wurden ohne weitere Aufarbeitung in einem Gaschromatographen oder in der GC—MS-Kombination analysiert.

Zur vollständigen Erfassung der Corticosteroide wurden auch die Methoximtrimethylsilylether hergestellt [16]: Dazu wurde ein anderer Teil des organischen Extraktes (siehe oben) in einem Schmelzpunktrörchen mit 20 ml einer gesättigten Lösung von Methoxylamin in Pyridin versetzt. Die Lösung wurde 15 min auf 75° erwärmt und dann im Vakuum eingedampft. Nach Zugabe von 5 ml N-Trimethylsilyl-N-methyl-trifluoracetamid wurde die Reaktionsmischung unter Luftabschluss 2 h bei 100° erhitzt und dann ohne weitere Reinigung in der Kombination analysiert.

Methode B. Stark wasserlösliche Steroide oder Steroidkonjugate wie Corticosteroide werden von der XAD-Säule beim Nachwaschen mit Wasser teilweise entfernt [17]. Da wir klären wollten, ob derartige Verluste auch bei einem Verzicht auf die XAD- und Sephadextrennung auftreten, wurde wie folgt aufgearbeitet:

· 2 l Hämofiltrat wurden mit Natriumacetat und Eisessig auf pH 4.7 und auf eine Pufferkonzentration von 0.5 Mol/l gebracht. Zu der Lösung wurden 10 ml Helicase hinzugefügt und bei 37° drei Tage verseift. Die freigesetzten Steroide wurden nach Horning und Pfaffenberger [13] extrahiert, zusätzlich wurden basiche Verbindungen mit zweimal 50 ml 0.1 *N* HCl, die 0.5 Mol NaCl enthielt, entfernt. Säuren wurden an DEAP-LH-20 [14], Cholesterin an Lipidex®-5000 [20 × 1 cm, 10 g Füllmaterial; Laufmittel; Methanol—Wasser—Chloroform (9:2:1), 0—30 ml] entfernt [18]. Die Probe wurde an-

schliessend an Kieselgel gereinigt [15] und derivatisiert.

Trennungsgang B hat den Vorteil, bei kleinerem Gesamtverlust und weniger Trennstufen schneller durchgeführt werden zu können, aber den Nachteil, dass die Steroidfraktion noch sehr viele Beimengungen enthält. Das Verfahren lässt sich daher nur anwenden, wenn die biologische Probe nahezu frei von Eiweiss ist. Eine auffällige Änderung der Zusammensetzung des Steroidprofils wurde nicht festgestellt.

Neuere Untersuchungen in unserem Arbeitskreis ergaben [19], dass nach der enzymatischen Verseifung beim Ausschütteln der Steroide mit organischen Lösungsmitteln und Nachwaschen mit wässrigen Lösungen erhebliche Verluste an Corticosteroiden auftreten. Diese Verluste manifestieren sich in den in dieser Arbeit reproduzierten Profilen, ändern jedoch nichts am Verhältnis der Androstane, das sich bei der Urämie entscheidend ändert.

Blutplasma

Zur Isolierung der Steroidsulfate aus Blutplasma wurden Volumina von 30—250 ml eingesetzt. Vor Verwendung der XAD-Säule wurde das Plasma mit physiologischer Kochsalzlösung auf das 10-fache verdünnt, um ein Ausfallen von Eiweiss zu vermeiden. Dann wurde analog wie bei der Aufarbeitung der Hämofiltrate verfahren (Methode A).

Urin

Die Urinsteroide wurde nach der Aufarbeitungsvorschrift von Horning und Pfaffenberger gewonnen [13]. Die eingesetzten Flüssigkeitsmengen betrugen 20—100 ml.

Verwendete Geräte

Gaschromatograph. Gaschromatograph Carlo Erba 2300 mit Glaskapillarsäulen ausgerüstet. Glaskapillarsäule nach statischer Methode mit SE-30-Film belegt [20, 21]. Injektortemperatur: 275°; Detektor: Flammenionisation (FID); Temperaturprogramm: 150—300°, 2°/min; Split: 1/20; Durchflussgeschwindigkeit (Helium): 2 ml/min.

Gaschromatograph—Massenspektrometer-Kombinationen. (1) CH-7 (Varian-MAT) mit gepackter Säule; Säule mit 3% SE-30 auf Supelcoport 100—120 mesh; Injektortemperatur 270°. Temperaturprogramm: 200—300°, 4°/min; Durchflussgeschwindigkeit (Helium): 20 ml/min. Separator: Biemann-Watson (zweistufig); Ionisierungsenergie: 70 eV. Die Kombination wurde mit einem Computer (Spectrosystem 100 MS; Varian 620/L) gekoppelt. (2) LKB 2091-Gerät mit Glaskapillarsäule (nach statischer Methode mit SE-30 belegt). Injektortemperatur: 275°; Temperaturprogramm: 150—300°, 2°/min; Durchflussgeschwindigkeit: 5 ml/min; Ionisierungsenergie: 70 eV. Registrierung des TIC bei 20 eV; LKB 2130-Datensystem mit PDP-11-05 Computer der Firma Digital Equipment.

Säulenmaterialien

XAD-4 (Serva, Heidelberg, B.R.D.); Sephadex-LH-20 (Pharmacia, Uppsala, Schweden); DEAP-LH-20 hergestellt aus Sephadex-LH-20 nach Almé und

Nyström [14]; Lipidex®-5000 (Packard, Zürich, Schweiz); Kieselgel (0.05—0.2 mm Durchmesser; E. Merck, Darmstadt, B.R.D.).

Chemikalien

Referenzsteroide: Makor (Jerusalem, Israel), Merck, Sigma (München, B.R.D.). Helicase: Boehringer (Mannheim, B.R.D.). N-Trimethylsilyl-N-methyl-trifluoracetamid: Macherey & Nagel (Düren, B.R.D.). Methoxyamin · HCl: Serva.

Retentionsindices

Die Retentionsindices der Steroidtrimethylsilylether wurden an dem Carlo-Erba-Gaschromatographen mit den oben angegebenen Daten unter Zusatz einer Mischung geradkettiger, unverzweigter Kohlenwasserstoffe von $C_{16}H_{34}$ bis $C_{36}H_{74}$ gemessen. Die Retentionsindices der Steroidtrimethylsilylether wurden auf die Kohlenwasserstoffe, zwischen denen der Peak des Trimethylsilylethers jeweils lag, bezogen.

Artefaktbildung

Corticosteroide werden thermisch aber auch beim Stehen in Lösungsmitteln leicht zu 17-Ketosteroiden abgebaut beziehungsweise in D-Homosteroide umgelagert [22]. Um eine derartige Artefaktbildung ermitteln zu können, wurden jeweils 17 mg der Corticosteroide Tetrahydrocortisol und Tetrahydrocortison in 100 ml Hämofiltrat als Matrix zugesetzt und der gesamte Trennungsgang durchgeführt. Der mögliche Abbau der Corticosteroide wurde dünnschicht- und gaschromatographisch sowie mit der GC—MS-Kombination verfolgt. Die Artefaktbildung unter den gewählten Bedingungen betrug im gesamten Trennungsgang ca. 2%.

ERGEBNISSE UND DISKUSSION

Fig. 1 zeigt das Glaskapillargaschromatogramm der Steroidsulfatfraktion aus Hämofiltrat eines männlichen Urämikers. Die Nummern über den Peaks in allen Gaschromatogrammen entsprechen den in der Tabelle I angeführten Steroiden, die durch Massenspektren und Retentionsindices identifiziert werden konnten. In Klammern stehen die Trivialnamen der Steroide. Steroidsulfatfraktionen aus den Hämofiltraten weiblicher Probanden lieferten ähnliche Profile.

Da das Hämofiltrat auch als "Primärharn" bezeichnet wird, sollte die Steroidzusammensetzung entweder Urin- oder Blutsteroidprofilen Nierengesunder ähnlich sein. Denkbar war auch eine Mischung beider Profile. Entgegen diesen Erwartungen enthalten die Hämofiltrate nur relativ kleine Mengen an Dehydroepiandrosteron, dem Hauptsteroid der Sulfatfraktion im Blut Nierengesunder, und an Androsteron und Etiocholanolon, den Hauptausscheidungsmetaboliten des Dehydroepiandrosterons im Urin Gesunder.

Als Hauptsteroide findet man im Hämofiltrat von Urämikern dagegen 11β-Hydroxyandrosteron und -etiocholanolon und 11-Ketoandrosteron und -etiocholanolon, die im Blut und Urin Nierengesunder nur in kleiner Menge vorhanden sind [23, 24].

Diese Befunde stehen im Einklang mit einer weit zurückliegenden Unter-

TABELLE I

NAMEN UND RETENTIONSINDICES DER IN DEN CHROMATOGRAMMEN GETRENNTEN STEROIDTRIMETHYLSILYLETHER

Nr.	Name des Steroids	Retentionsindex
1	3α-Hydroxy-5-androsten-17-on	2450
2	3α-Hydroxy-5α-androstan-17-on (Androsteron)	2472
3	3α-Hydroxy-5β-androstan-11,17-dion (11-Ketoetiocholanolon)	2499
P	Dioctylphthalat	2510
4	3β-Hydroxy-5-androsten-17-on (Dehydroepiandrosteron)	2550
5	3α, 17β-Dihydroxy-5β-androstan	2560
6	3α-Hydroxy-5β-androstan-11,17-dion (11-Ketoetiocholanolon)	2566
7	3β-Hydroxy-5α-androstan-17-on (Epiandrosteron)	2570
2′	Enolether von 2	2566
3′	Enolether von 3	2570
4′	Enolether von 4	2620
8	3β,17β-Dihydroxy-5-androsten (Androstendiol)	2638
9	3β,17β-Dihydroxy-5α-androstan	2644
10	3α,11β-Dihydroxy-5α-androstan-17-on (3-Trimethylsilylether) (11β-Hydroxyandrosteron)	2652
11	3,16-Dihydroxyandrostan-17-on	2652
6′	Enolether von 6 (3,17-di-Trimethylsilylether)	2660
12	3α,11β-Dihydroxy-5β-androstan-17-on (3-Trimethylsilylether) (11β-Hydroxyetiocholanolon)	2664
13	3α-Hydroxy-5α-androstan-11,17-dion (3,17-di-Trimethylsilylether) (11-Ketoandrosteron)	2668
14	Dihydroxyandrostanon	2684
15	3,16,17-Trihydroxyandrostan	2688
12′	Enolether von 12 (3,11-di-Trimethylsilylether)	2698
16	3α,18-Dihydroxyandrostan-17-on	2706
17	3β-Hydroxy-5-pregnen-20-on (Pregnenolon)	2708
18	3β,16α-Dihydroxy-5-androsten-17-on (16α-Hydroxydehydroepiandrosteron)	2735
19	3,16,17-Trihydroxyandrostan	2758
20	3,16,17-Trihydroxyandrostan	2764
21	3α,20α-Dihydroxy-5β-pregnan (Pregnandiol)	2766
22	3β,16β,17α-Trihydroxy-5-androsten	2783
23	3β,20α-Dihydroxy-5-pregnen (Pregnendiol)	2837
24	3β,16α,17β-Trihydroxy-5-androsten (Androstentriol)	2870
25	3,16,17-Trihydroxyandrosten	2880
26	3,20-Dihydroxypregnan	2891
27	3,16,20-Trihydroxy-5-pregnen	2905
28	3α,17α,20α-Trihydroxy-5β-pregnan (Pregnantriol)	2948
29	3,17,20-Trihydroxypregnan	2968
30	3β,17α,20α-Trihydroxy-5-pregnen	3012
31	3,17,20-Trihydroxypregnan-11-on	3030
32	3,11,17,20-Tetrahydroxypregnan	3102
33	3α,17α,21-Trihydroxy-5β-pregnan-11,20-dion (Tetrahydrocortison)	3110
34	3α,17α,21-Trihydroxy-5α-pregnan-11,20-dion (α-Tetrahydrocortison)	3123
35	3β-Hydroxy-5-cholesten (Cholesterin)	3138
36	3α,11β,17α,21-Tetrahydroxy-5β-pregnan-20-on (Tetrahydrocortisol)	3142
37	3α,11β,17α,21-Tetrahydroxy-5α-pregnan-20-on (α-Tetrahydrocortisol)	3175

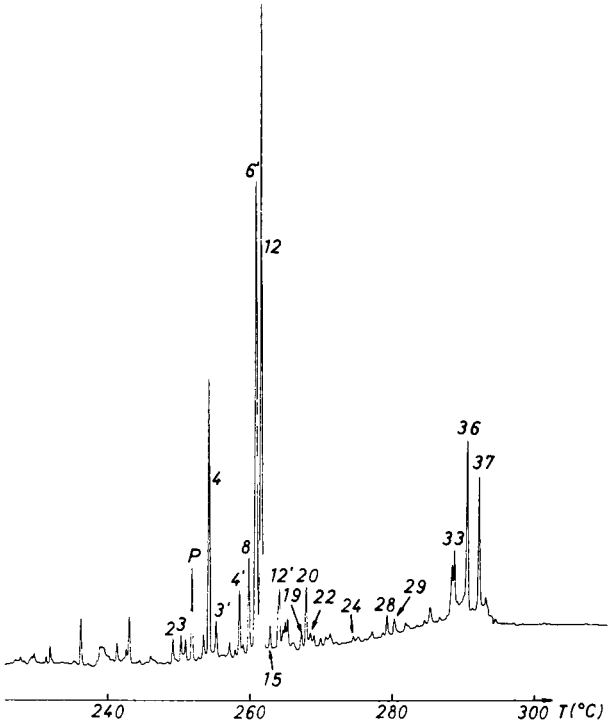

Fig. 1. Glasskapillargaschromatogramm der Steroidtrimethylsilylether der Sulfatfraktion aus Hämofiltrat eines Urämikers. 30 m Glaskapillarsäule, SE-30; Temperaturprogramm: 150—300°, 2°/min.

suchung von Savard [25], der nach Aufarbeitung von 100 l Dialysat papierchromatographisch diese vier Steroide nachgewiesen hat. Offenbar geriet diese Beobachtung jedoch bald in Vergessenheit.

Die 11-oxidierten Androstane sind Abbauprodukte von Corticosteroiden [25—27]. Die Corticosteroide Tetrahydrocortison, α-Tetrahydrocortison, Tetrahydrocortisol und α-Tetrahydrocortisol wurden im Blut von Urämikern mit Radioimmunoassaymethoden in 10-fach erhöhter Menge gefunden, dagegen wurde im Urin von Nierenkranken nur 1/10 der normalen Ausscheidungsrate nachgewiesen. Der Befund wurde theoretisch zu deuten versucht [28]. Diese Corticosteroide wurden ebenfalls im Dialysat von Savard [25] identifiziert, auch er fand eine auffällig geringe Konzentration.

Bemerkenswerterweise sind im Hämofiltrat hauptsächlich Steroide mit hydriertem B-Ring vorhanden, während im Plasma Steroide mit Δ-5-Doppelbindung vorherrschen. In dieser Hinsicht gleichen die Hämofiltratsteroide eher Urinsteroiden, bei denen ebenfalls B-Ring-gesättigte Steroide überwiegen.

Die Glucuronidfraktionen enthalten ebenfalls als Hauptsteroide in Stellung 11 sauerstoffsubstituierte Androstane. Freie Steroide lassen sich mit unserem Untersuchungsverfahren im Hämofiltrat ohne erhebliche Vergrösserung des Ausgangsvolumens nicht nachweisen, ihre Konzentrationen liegen unterhalb unserer Erfassungsgrenze.

Die von allen Erwartungen stark abweichende Zusammensetzung der Ste-

roidfraktionen des Hämofiltrats liess es wünschenwert erscheinen, auch Steroidplasmaprofile von Urämikern zu untersuchen.

Da die Aufnahme eines Plasmasteroidprofils ein Mindestvolumen von 20—30 ml Plasma, entsprechend 40—60 ml Blut, notwendig macht [29] und Urämikern die Abnahme dieser Blutmengen nicht zuzumuten ist, wurde jeweils Plasma von Männern und Frauen gepoolt und untersucht. Das Glaskapillargaschromatogramm des Patientenpools (Fig. 2) zeigt gegenüber dem Steroidprofil des gesunden Mannes (Fig. 3) drastische Änderungen. Der Anteil des Dehydroepiandrosterons ist im Patientenpool gleich oder sogar kleiner als der seines Metaboliten, des Androstendiols.

Um abzuklären, ob Änderungen des Steroidstoffwechsels bereits vor dem völligen Versagen der Nierenfunktionen festgestellt werden können, untersuchten wir Urinsteroidprofile von Patienten mit eingeschränkten Nierenfunktionen in verschiedenen Krankheitsstadien. Einige dieser Patienten wurden bereits hämofiltriert, waren aber noch in der Lage, Urin abzupressen.

Je nach fortgeschrittenem Krankheitszustand weisen die Steroidprofile dieser Patienten einen Anstieg 11-oxidierter Androstane auf (Fig. 4). Die Ausscheidungsrate von Androsteron und Etiocholanolon ist demgegenüber rückläufig.

Auffällig ist bei männlichen Patienten das Verhältnis von Androsteron zu Etiocholanolon: Während normale Männer, von wenigen Ausnahmen abgesehen, eine deutlich grössere Ausscheidung von Androsteron als Etiocholanolon zeigen und dies als geradezu charakteristisch gewertet wurde [13], fanden wir in 5 von 7 untersuchten Fällen ein Ausscheidungsverhältnis von Androsteron zu Etiocholanolon, wie man es sonst nur bei gesunden Frauen findet [13]. Einer der beiden Patienten, die eine "normale" Androsteron—Etiocholanolon-Ausscheidung zeigten, befand sich erst im Anfangsstadium der Krankheit. Auch bei nierenkranken Frauen wurde eine Erhöhung des Etiocholanolonspiegels im Urin gegenüber Gesunden beobachtet.

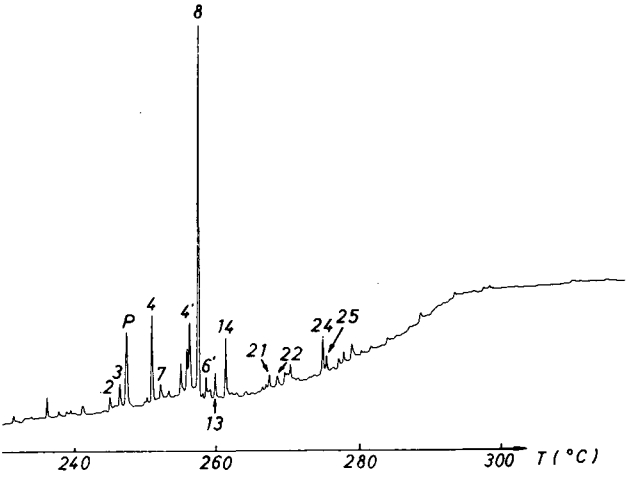

Fig. 2. Gaschromatogramm der Steroidtrimethylsilylether der Sulfatfraktion aus Plasma männlicher Urämiker. GC-Bedingungen wie in Fig. 1.

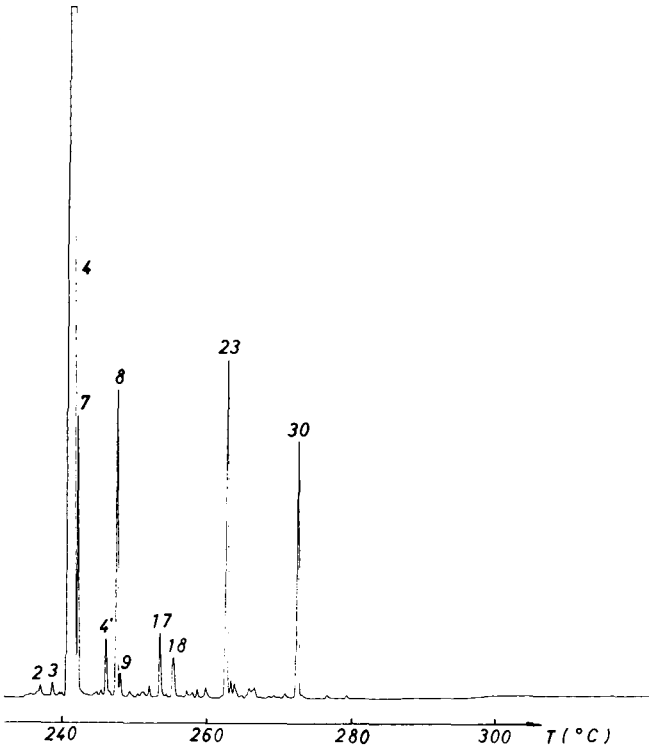

Fig. 3. Gaschromatogramm der Steroidtrimethylsilylether der Sulfatfraktion aus Plasma eines gesunden Mannes. GC-Bedingungen wie in Fig. 1.

DANK

Diese Arbeit wurde aus Mitteln der Deutschen Forschungsgemeinschaft, des Fonds der Chemischen Industrie und der Robert-Pfleger-Stiftung gefördert. Herrn Dr. J. Reiner danken wir für die Herstellung der Glaskapillarsäulen und Herrn M. Glaessner für zahlreiche Messungen an der Kombination Gaschromatograph-Massenspektrometer.

ZUSAMMENFASSUNG

Steroidprofile der Sulfatfraktionen aus Hämofiltrat von Urämikern enthalten als Hauptsteroide 11β-Hydroxyetiocholanolon, 11-Ketoetiocholanolon, 11β-Hydroxyandrosteron und 11-Ketoandrosteron. Im Blut von Urämikern ist Androstendiol Hauptsteroid der Sulfatfraktion, während im Blut Gesunder Dehydroepiandrosteron Hauptsteroid ist. Allmähliches Nierenversagen zeigt sich durch einen Anstieg der 11-oxidierten Androstankonjugate im Urin.

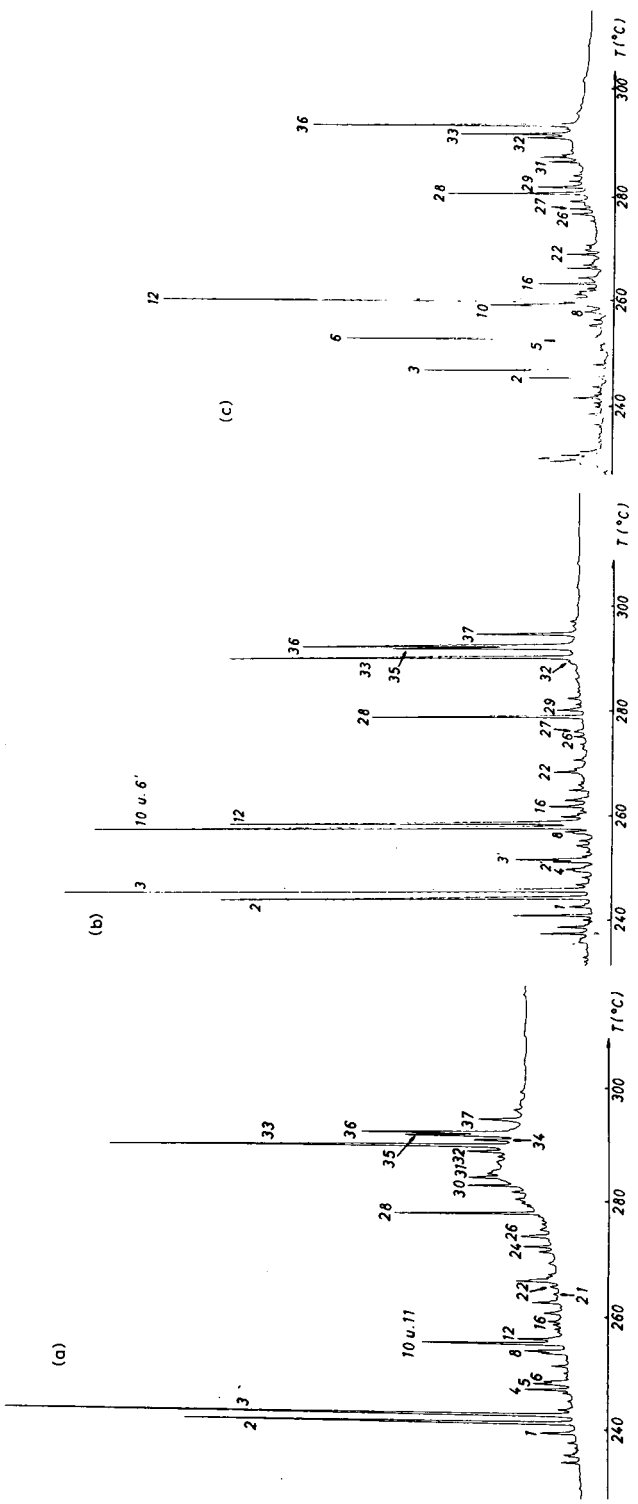

Fig. 4. Gaschromatogramme der Steroidtrimethylsilylether der Steroide aus Urin männlicher Patienten mit eingeschränkten Nierenfunktionen. GC-Bedingungen wie in Fig. 1. (a) Anfangsstadium; (b) fortgeschrittenes Stadium; (c) unmittelbar vor Hämofiltration.

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Biomedical Applications

CHROMBIO. 201

COMPARATIVE DETERMINATION OF PLASMA CHOLESTEROL AND TRIACYLGLYCEROL LEVELS BY AUTOMATED GAS—LIQUID CHROMATOGRAPHIC AND AUTOANALYZER METHODS

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SUMMARY

Plasma samples obtained during a prevalence study of hyperlipemia in a free living urban population were analyzed for total cholesterol and triacylglycerol content by automated high-temperature gas—liquid chromatographic (GLC) and automated colorimetric (Auto-Analyzer, AA11) methods. The analyses were done over a three-year period. The methods gave excellent overall correlation for both total cholesterol (r = 0.9811) and total triacylglycerols (r = 0.9739). Detailed comparisons of the results obtained by the two methods with natural samples over the entire concentration range, indicated that the GLC method gave cholesterol values 5—10 mg% lower and triacylglycerol values 10—20 mg% lower than the corresponding AA11 determinations. The differences between the two methods are attributed to an overestimation of the cholesterol and triacylglycerol levels by the AA11 method due to presence of variable amounts of interfering chromogens in the plasma extracts. The between-method relative error ranged from 3 to 5% for cholesterol and from 5 to 10% for triacylglycerols. The within-day standard deviation of GLC averaged 2.3 mg% for cholesterol and 3.5 mg% for triacylglycerols. The between-day standard deviation of the GLC method averaged about 6 mg% for both cholesterol and triacylglycerols. The within-day, within GLC, relative error averaged 1.12% for cholesterol and 2.66% for triacylglycerols. The apparent high precision and high accuracy of the GLC method recommend it as an alternative to the indirect methods of plasma cholesterol and triacylglycerol analysis, especially where a smaller throughput of samples is not a limitation and where both total amount and composition of the lipids is of interest.

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INTRODUCTION

High-temperature gas—liquid chromatography (GLC) is a sensitive and rapid technique by which neutral plasma lipids can be separated into individual lipid classes or molecular species [1—5] and accurate identification and quantitation obtained for each component. We have recently demonstrated that this method of total plasma lipid analysis can be automated and that the results obtained for total cholesterol and total triacylglycerols compare favourably to those realized with colorimetric methods, when tested with reference standards [4]. In the present study we have compared the results of the GLC and the AutoAnalyzer methods for analysis of total cholesterol and triacylglycerols in over 1000 samples of plasma from a free living urban population. In general the GLC method gave values which were 5—20 mg% below those of the AutoAnalyzer method [6]. The discrepancies are traced to various inherent errors and biases in the GLC and AutoAnalyzer methods.

MATERIALS AND METHODS

The standard acylglycerol, free cholesterol and cholesteryl ester mixtures employed in the study were prepared in the laboratory from chromatographically pure (99% + single components) materials supplied by Serdary Research Labs. (London, Canada) and the Applied Science Labs. (State College, Pa., U.S.A.). BDH Control Sera were obtained from BDH (Toronto, Canada). To each vial of the latter containing the freeze-dried solids from 10 ml plasma, 10 ml of distilled water were added to obtain complete solution. Other control plasma samples of known content of total cholesterol and total triacylglycerol (samples LRC 1, LRC 2, and LRC 3), and the unknown samples from a population survey were supplied by the Toronto-McMaster Lipid Research Clinic (University of Toronto, Toronto, Canada). The unknown plasma samples had been prepared from fresh blood collected in ethylene diaminetetraacetate-containing vials, and were stored in a frozen state at —20° for a maximum of 3 months before analysis. Prior to withdrawal of any aliquots the thawed samples were thoroughly shaken to avoid concentration gradients [7]. Phospholipase C (α-toxin of *Clostridium welchii*) was purchased from Sigma (St. Louis, Mo., U.S.A.). Trisil—BSA reagent was supplied by Pierce (Rockford, Ill., U.S.A.). Other reagents and solvents were of Fisher certified-reagent grade and were tested for lipid contaminants prior to use.

Preparation of samples for analysis

EDTA (0.01%)—plasma (0.2—0.5 ml) was added to a PTFE-lined screw-cap centrifuge tube (18-ml capacity) containing 0.2—0.4 mg phospholipase C in 4 ml of 17.5 m*M* Tris buffer, pH 7.3, along with 1.3 ml of 1% $CaCl_2$ and 1 ml of diethyl ether, and the mixture incubated with shaking for 2 h at 30°. The reaction mixture was then treated with 5 drops of 0.1 *N* HCl and extracted once by vigorous shaking with 10 ml of chloroform—methanol (2:1) containing 150—250 μg tridecanoylglycerol as internal standard. The solvent phases were separated by centrifuging for 10 min at 200 *g*. The clear chloroform phase was removed from the bottom of the tube and was dried by passing

through a Pasteur pipet containing 2 g of anhydrous sodium sulphate. The effluent was evaporated under nitrogen and the residue dissolved in Trisil—BSA (150—250 μl) and transferred to a sampling vial, and the vial sealed.

Gas chromatographic methods

The automated high-temperature GLC analysis was performed on a Hewlett-Packard Model 5700 A automatic gas chromatograph equipped with dual stainless-steel columns (50 cm × 2 mm I.D.) containing 3% OV-1 on 100—120 mesh Gas-Chrom Q (Applied Science Labs.) and an automatic liquid sample injector (Model HP 7671 A), as previously described [4]. The GLC separations were routinely made by temperature programming from 175 to 350° at either 4 or 8°/min with the columns in the dual compensating mode and dry nitrogen as the carrier gas (40 ml/min).

After every 200 analyses the first 1—2 in. of the column packing were replaced with fresh packing and the columns reconditioned at 350° with the normal carrier gas flow. Silicone oxide deposits, which accumulated on the detector due to injection of the silylation mixture, were routinely removed by scrubbing with chloroform every two weeks.

The integrator output was simultaneously recorded on a paper chart and on a punched paper tape indicating the tube number, the peak retention time and area in a computer compatible ASC 11 code, which is a basic language program for off-line data processing. The punched tape record was processed using modifications of the computer programs provided by Hewlett-Packard (CALIST, CALIB and HP7600), as previously described [4]. The peak areas for the free cholesterol and cholesteryl esters, and the triacylglycerols, respectively, were summed using appropriate calibration and conversion factors to provide estimates for total-plasma cholesterol and triacylglycerols. It was noted (see Results) that the integrator record could be quite erratic for certain slope sensitivity settings and that it required a systematic examination for errors in baseline resetting if precise results were to be obtained. The absolute amounts of plasma lipids were quantitated by means of an internal standard (tridecanoylglycerol) added to the plasma at the time of lipid extraction at a relative proportion of 10—20% of total. The quality of the analytical results was controlled by systematic monitoring of a synthetic and a natural plasma external reference standard, which were analyzed simultaneously with any unknown samples.

AutoAnalyzer methods

The colorimetric analyses were performed with an AutoAnalyzer AA11 (Technicon, Tarrytown, N.Y., U.S.A.) instrument. The estimates for total cholesterol and total triacylglycerols were obtained on Zeolite-treated isopropanol extracts as outlined in the Manual of Laboratory Operations, Lipid Research Clinics Program [8]. Pure cholesterol and trioleoylglycerol (triolein) standards and a serum calibrator were supplied by the Lipid Standardization Laboratory (Center for Disease Control, Atlanta, Ga., U.S.A.). Each AA11 run was initially set up and checked with free cholesterol standards. Subsequently the output was adjusted downward on the basis of a daily analysis of a cholesterol serum calibrator with a cholesterol value determined by the

method of Abell and Kendall as modified by the Lipid Standardization Laboratory [8]. When a step-up series of cholesterol standards was analyzed, the AA11 generally gave a cholesterol value of 325 ± 5 mg% for a serum calibrator with a target value of 296. The triacylglycerols were determined by color development with the glycerol liberated from the neutral lipid extract of plasma upon saponification. The glycerol yield was expressed as mg% of trioleoylglycerol.

Statistical analysis

The evaluation of the GLC procedure for cholesterol and triacylglycerol determination was modeled on a comparable study of methodology reported by Lippel et al. [9]. Systematic errors were measured by the difference between average GLC values and the AA11 or target values by a bias statistic. The magnitude of random errors was measured by the variance or standard deviation. The relative error values are averages of percent deviations defined as:

$$\text{Relative error} = \frac{\text{GLC value} - \text{AA11 Value}}{\text{AA11 value}} \times 100$$

A coefficient of variation between duplicates was calculated using the formula

$$\text{C.V. \%} = \frac{100\sqrt{d^2/2}}{\bar{x}}$$

where d is the difference between duplicates and $\bar{x}$ is the mean.

The within-day standard deviation was used as the measure of within-day variability. The overall standard deviation was used as the measure of the variability of a single determination of a quality control sample by the GLC method. A correlation coefficient and a regression coefficient for the data were calculated according to established statistical procedure [10]. A Hewlett-Packard HP-9821A programmable calculator was used to assist in these determinations.

RESULTS

The overall analytical routine was tested with standard mixtures of neutral lipids prepared in the laboratory and with standard plasma lipid samples purchased commercially or acquired from the Lipid Research Clinics Program and satisfactory results were obtained as previously described [4]. It remained to be demonstrated that such analyses could be performed routinely on a large number of unknown samples and to establish how the GLC values compared to those obtained by the AutoAnalyzer method generally employed for the determination of plasma total cholesterol and triacylglycerols in clinical laboratories.

Acquisition of data

Following the preliminary enzymic and chemical modification of the plasma lipids, the quantitative estimates of plasma total cholesterol and triacylglycerols

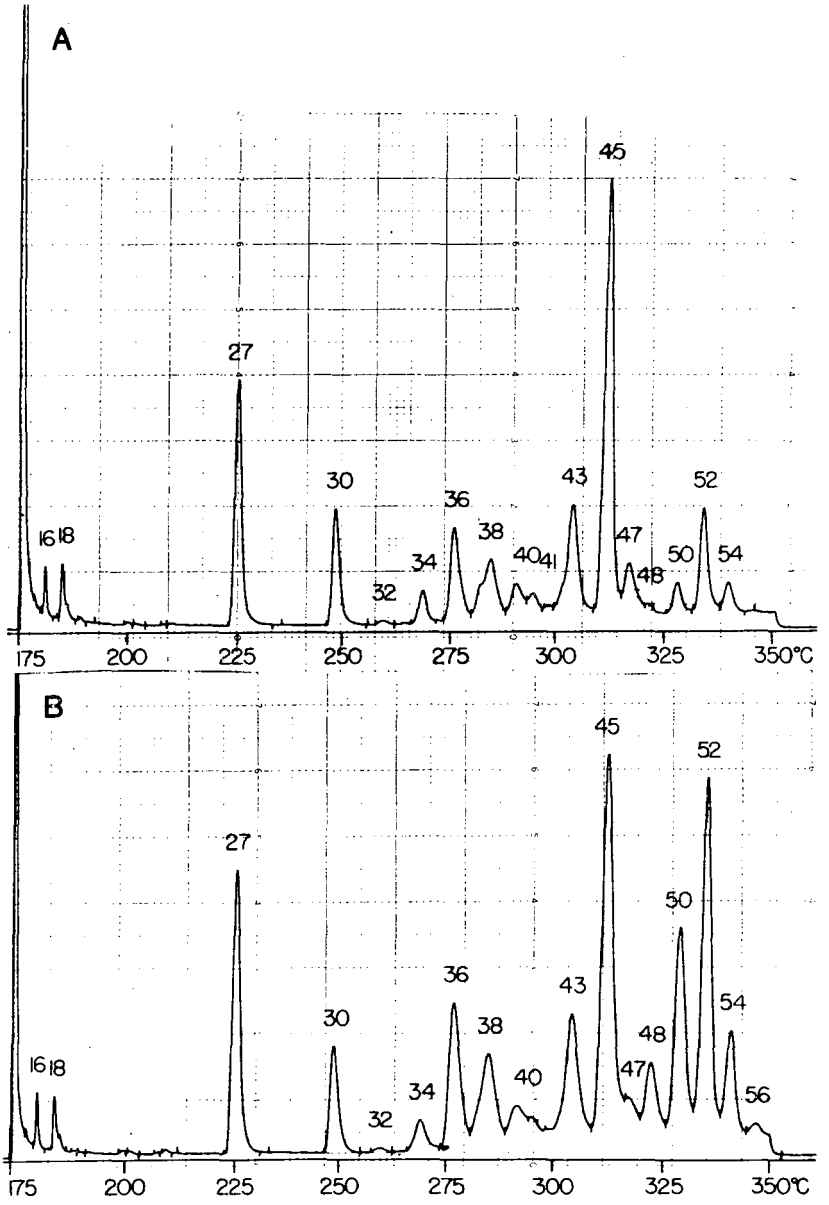

Fig. 1. Total lipid profiles (A) a normolipemic and (B) a hyperlipemic plasma as obtained using a low-temperature gradient GLC. Conditions of high-temperature GLC as given in text. Peaks 16 and 18, trimethylsilylesters of free fatty acids with 16 and 18 acyl carbons; peak 27, trimethylsilylether of cholesterol; peak 30, tridecanoylglycerol internal standard; peak 34, trimethylsilylether of palmitoylsphingosine; peaks 36—42, trimethylsilylethers of diacylglycerols of a total number of 34—40 acyl carbons; peaks 43—47, cholesteryl esters of fatty acids with a total number of 16—20 acyl carbons; peaks 48—56, triacylglycerols with a total number of 48—56 acyl carbons. Sample size: 1 μl of an approximately 1% solution in silylation mixture. Attenuation: 100 times full sensitivity.

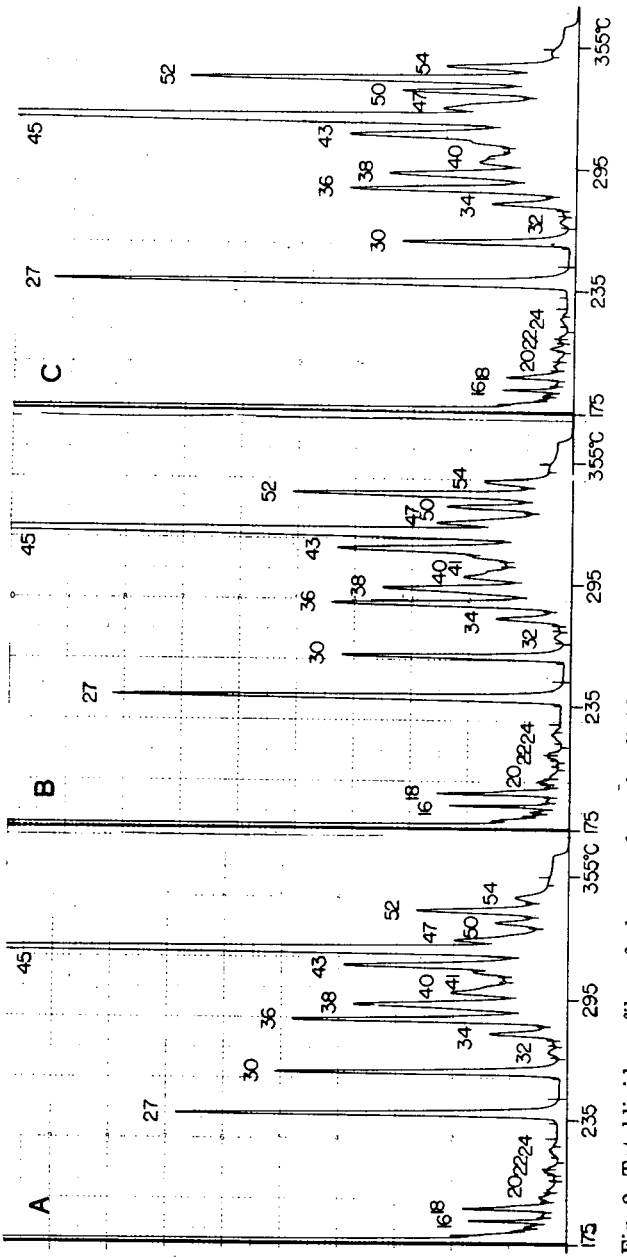

Fig. 2. Total-lipid profiles of plasma of varying lipid content as obtained by a high-temperature gradient GLC. Conditions of high-temperature GLC as given in text. Peak identification as in Fig. 1.

were obtained by GLC using two different rates of temperature programming. For optimum resolution of peaks the column temperature was programmed in the range 175—350° at a rate of 4°/min. Fig. 1 shows representative total plasma lipid profiles of a normal subject and a patient with hyperlipoproteinemia. It is seen that the peaks for free cholesterol, the tridecanoylglycerol internal standard, and the various molecular species of the cholesteryl esters and triacylglycerols are clearly resolved for both the normolipemic and the hyperlipemic plasma, although some overlap may occur for the cholesteryl esters and triacylglycerols in the hyperlipemic plasma. The partial overlap of the cholesteryl esters and triacylglycerols may be avoided by appropriate dilution of the sample and suitable adjustment in the amount of the added internal standard. The overlapping of the cholesteryl esters and triacylglycerols is much more serious and cannot be avoided by dilution of the sample when it is due to high levels of short-chain fatty acids in the triacylglycerol fraction. Under normal conditions, however, short-chain fatty acids are largely absent from plasma triacylglycerols and carbon numbers of C_{42}—C_{46} usually make up only a minor proportion of the total mass of plasma triacylglycerols. It should also be noted that in all instances the baseline elevation due to column bleed has been minimal, as indicated by the small difference between the final baseline elevation and the point of the last baseline reset (usually between the peaks for free cholesterol and the tridecanoylglycerol). Despite the excellent peak resolution, this program failed to give a reliable peak area quantitation by automated integration due to erratic baseline reset by the peak slope sensor (see below).

Fig. 2 gives the total lipid profiles of three plasma samples of varying total lipid content as obtained in the temperature range 175—350° using a heating rate of 8°/min. Under these conditions the slopes of the peaks are much steeper and the frequency of erratic baseline resetting much lower. However, there is also much more peak overlapping than when the lower rate of temperature programming is employed. Nevertheless, the major chemical classes and molecular species of cholesteryl esters and acylglycerols are reasonably well resolved so that precise and accurate quantitation of peak areas may be expected. Furthermore, a faster program rate improves the detection and quantitation of the peak areas due to minor components (monoacylglycerols). The faster program rate also shortens the overall time of analysis and thereby minimizes the decomposition of the more sensitive components and increases the overall efficiency of the operation of the analytical system. With proper peak area measurements both temperature programming rates gave comparable quantitative results, but the esthetic impression was more favourable with the slower heating rate.

In both instances the total cholesterol value was obtained by adding the peak areas for the free cholesterol and the various cholesteryl esters using appropriate calibration factors and molar conversion ratios. The total triacylglycerols were calculated by summing the peak areas in the range C_{48}—C_{56} using appropriate peak area correction factors. Alternatively the peak areas were summed over a range of preselected elution times (windows) and overall correction factors applied [4]. This method avoided the need for

TABLE I

FREQUENCY OF DIFFERENT ERRORS IN AN AUTOMATED GLC ANALYSIS OF TOTAL LIPIDS OF FROZEN PLASMA SAMPLES

Number of samples	Error		
	Faulty integration	Copying error	Non-representative sampling*
Slow temperature program (4°/min)			
Batch of 800	130	10	40
Batch of 250	14	2	4
Batch of 400	45	6	20
Total 1450	189	18	66
Fast temperature program (8°/min)			
Batch of 197	8	2	5
Batch of 400	23	2	12
Batch of 100	2	0	3
Total 697	32	4	20

*Non-representative sampling due to incomplete dissolution of precipitate in frozen plasma samples.

accurate peak identification and the use of specific correction factors, which was not always possible to obtain.

Table I gives the frequency of erratic resetting of baseline by the electronic peak area integrator. With the slower temperature program over 10% of the runs contained baseline resetting errors. This problem was corrected to large extent (less than 5% error) by substituting a faster temperature program, which produced steeper peak slopes. The faster program also appears to have decreased the other errors, but this was due to greater care taken in sample handling and in data recording in the more recent experiments.

Precision of analyses

A measure of the precision or reproducibility of the GLC method of determining total cholesterol and total triacylglycerols in unknown plasma was obtained by calculation of the standard deviation and the coefficient of variation on repeat analyses at several levels of concentration of plasma lipids. Table II gives the mean values, standard deviations and coefficients of variation for 4 repeat analyses for total cholesterol and total triacylglycerols from 17 random samples of plasma. The overall coefficients of variation are 1.12 and 2.66% for the total cholesterol and total triacylglycerols, respectively, The reproducibility of these values or the precision of the GLC analysis itself for the natural samples is therefore of the same order as that previously observed for standard free cholesterol and triacylglycerols by this method [4]. Table III gives the range of values, mean of values, standard deviations and the

TABLE II

PRECISION OF QUADRUPLICATE REPEAT GLC ANALYSES OF PLASMA TOTAL CHOLESTEROL AND TRIACYLGLYCEROLS

Each sample was injected four times into the gas chromatograph in four cycles over a period of two days. C.V. = coefficient of variation; S.D. = standard deviation.

Sample	Cholesterol		Triacylglycerol	
	Mean ± S.D.	C.V. (%)	Mean ± S.D.	C.V. (%)
1	187.8±0.64	0.34	31.7±1.0	3.2
2	182.5±4.5	2.5	32.8±1.3	4.1
3	168.0±1.2	0.70	18.2±0.30	1.6
4	151.3±0.75	0.50	38.5±1.15	3.0
5	140.5±1.4	1.00	48.1±2.5	5.2
6	143.9±0.55	0.38	58.1±0.85	1.5
7	132.4±1.6	1.2	35.7±2.3	6.3
8	130.6±3.0	2.3	50.9±0.83	1.6
9	129.1±0.67	0.52	41.1±0.89	2.2
10	156.3±1.9	1.2	42.9±0.65	1.5
11	132.1±0.3	0.23	52.9±0.48	0.90
12	154.0±2.6	1.7	57.4±1.7	3.0
13	158.4±0.13	0.08	53.7±0.59	0.93
14	143.9±0.48	0.34	36.3±0.93	2.5
15	140.5±4.3	3.0	65.5±1.1	1.7
16	138.5±3.5	2.5	50.4±1.8	3.6
17	130.9±0.64	0.49	36.2±0.82	2.3
Average	148.2±1.66	1.12	45.1±1.12	2.66

coefficients of variation for the within-day variation of the estimates of total cholesterol and triacylglycerols as obtained on three samples of standard plasma of markedly different total lipid content following decaplicate repetition of the entire analytical routine on each sample, but excluding major adjustments in instrumentation. The overall coefficients of variation obtained in this instance are 1.14 and 1.93% for total cholesterol and total triacylglycerols, respectively. These values again are of the order obtained on repeat injections of the same sample. Table IV gives the range of values, the means and the standard deviations of the within-day and between-day variation observed for the entire GLC method when major instrument adjustments are also included. These variations were recorded for an external quality control standard over a 60-day period. It is seen that the within-day standard deviations of 2.2 and 3.0 mg% for total cholesterol and total triacylglycerols, respectively, are somewhat higher than those observed for the within-day repeat injections of the same sample or for repeat processing and analysis of the same sample including appropriate correction factors (Tables II and III, respectively). However, the between-day variation was highly significant and emphasized the need for the external standard for quality control of the analyses. When the unknown values are corrected for the day-to-day variation of the external reference standard, the standard deviations of the day-to-day variation become of the order of those seen for the within-day varia-

TABLE III

WITHIN-DAY VARIATION OF THE ENTIRE GLC METHOD OF ANALYSIS OF TOTAL CHOLESTEROL AND TOTAL TRIACYLGLYCEROLS ON REFERENCE PLASMA SAMPLES

The individual samples were pools of plasma from normolipemic and hyperlipemic subjects by the Toronto-McMaster Lipid Research Clinic

Plasma samples	Range (mg%) of decaplicate analyses		Mean values (mg%) ± S.D. of decaplicates		Coefficient of variation of decaplicates (%)	
	Cholesterol	Triacylglycerols	Cholesterol	Triacylglycerols	Cholesterol	Triacylglycerols
LRC 1	177—188	72—78	180.30±3.09	75.181±1.83	1.71	2.43
LRC 2	258—264	150—157	260.90±2.46	153.50 ±2.76	0.94	1.80
LRC 3	374—382	270—281	378.56±2.96	276.40 ±4.27	0.78	1.55
Average					1.14	1.93

TABLE IV

WITHIN-DAY AND DAY-TO-DAY VARIATION OF THE ENTIRE GLC METHOD OF ANALYSIS OF TOTAL CHOLESTEROL AND TOTAL TRIACYLGLYCEROLS IN A QUALITY CONTROL SAMPLE

The days of analyses were taken from a 60-day period in January, February and March of 1976. In addition to the entire routine of sample preparation, the method included bi-weekly detector cleaning, one column replacement, one detector jet replacement and one replacement of septum.

Days of analysis	Total cholesterol (mg%)		Total triacylglycerols (mg%)	
	Range	Mean ± S.D.	Range	Mean ± S.D.
1	153.6—155.7	154.9±0.85	87.6—89.4	88.4±0.75
2	168.6—173.0	170.2±2.33	89.1—96.4	92.5±2.75
3	156.0—159.3	156.2±2.18	83.6—94.0	88.3±3.98
4	161.4—166.0	163.5±2.11	95.8—97.6	96.8±0.84
5	152.6—160.4	154.9±3.63	91.6—106.3	97.7±6.5
6	161.7—165.5	164.6±3.01	99.4—114.0	108.6±5.13
7	166.4—178.7	169.6±2.3	95.7—106.5	102.7±4.6
8	170.0—171.7	171.8±1.34	101.2—104.8	103.3±1.74
9	164.6—169.7	168.7±2.88	96.5—101.7	93.3±2.24
10	166.9—170.5	168.7±1.42	101.1—104.1	102.7±1.35
11	168.3—171.7	169.6±1.42	98.5—102.2	100.8±1.60
12	165.0—170.7	168.3±2.29	96.5—106.7	103.1±4.21
Overall Mean		165.1		98.2
Total S.D.		6.3		6.5
Within-day S.D.		2.2		3.0
Between-day S.D.		6.3 ($P < 0.01$)		6.2 ($P < 0.01$)

tions. On the basis of these data it is concluded that the maximum error observed with the GLC method does not exceed 5%, which is about twice the coefficient of variation of repeat injections.

Fig. 3 shows a plot of the GLC results of duplicate analyses of 227 random samples of plasma for total cholesterol as obtained by analyses extending over a period of two years. A similar plot for total triacylglycerols is shown in Fig. 4. With few exceptions, only one determination was made per sample. However, all the GLC elution patterns were examined for errors in baseline resetting and where necessary, the peak areas were recalculated by cutting out and weighing the paper obtained by xeroxing the appropriate parts of the GLC records. A total of 56 runs were corrected in this way. There is an excellent agreement between the corresponding values, over 90% of which are found to be within ±10% of the ideal correlation line with intercept 0 and slope equal to 1.0. The average A and B values are 200.7±49.5 and 198.4±48.7 mg% for total cholesterol and 139.5±120.4 and 138.4±110.0 mg% for total triacylglycerols, respectively. The correlation coefficients for the cholesterol and triacylglycerol analyses of the A and B samples were 0.9348 and 0.9790, respectively. A calculation of the coefficient of variation of the duplicates gave values of 5.0 and 10.5% for the A and B samples of total cholesterol and triacylglycerols, respectively. These coefficients of variation are consid-

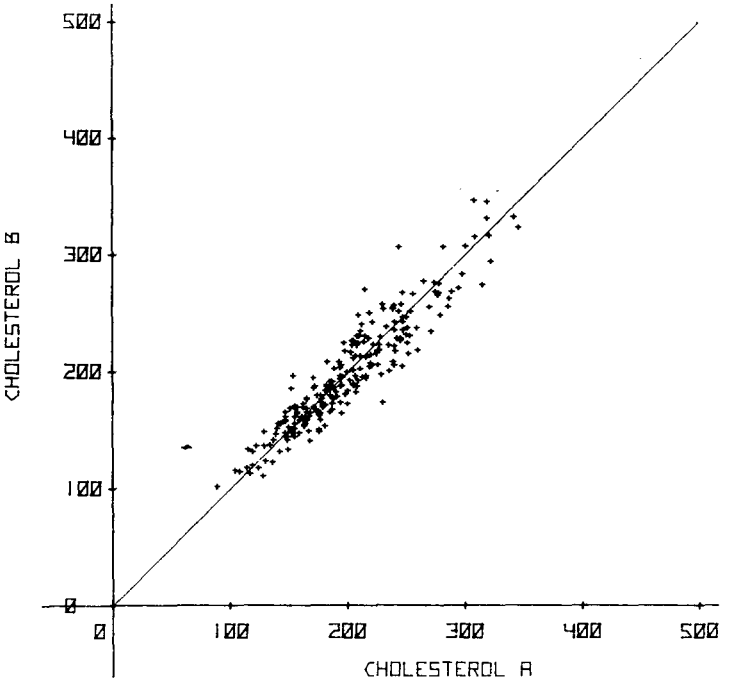

Fig. 3. Comparison of results (mg%) obtained by GLC for duplicate samples of plasma over a period of two years.

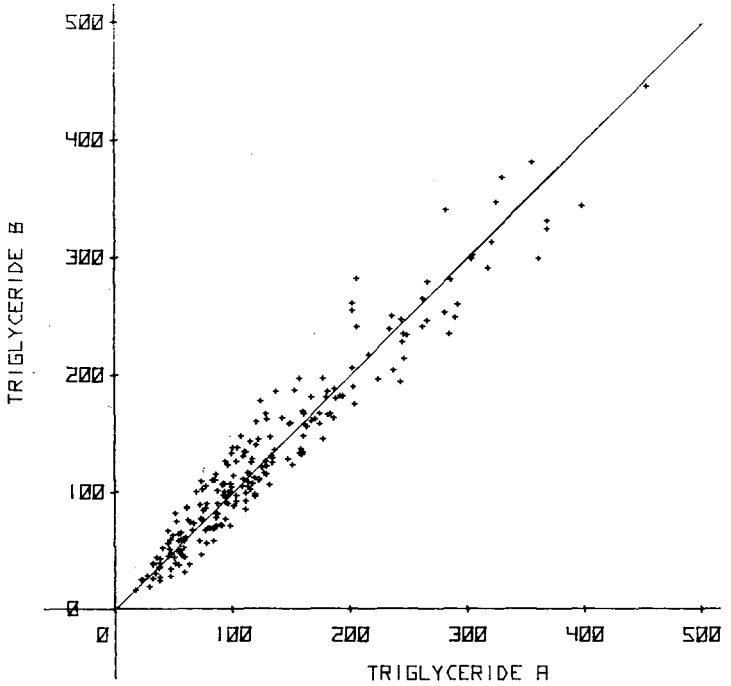

Fig. 4. Comparison of results (mg%) obtained by GLC for duplicate samples of plasma over a period of two years.

erably higher than those of the relative errors calculated from the multiple repeat analyses carried out over a shorter period of time. Nevertheless, it is obvious that the analytical system reproduces its results with high precision, as already noted for a manual version of the method in triacylglycerol analyses [11].

Bias

Any bias in the GLC analysis was assessed by comparing the results with the AutoAnalyzer target values. Table V gives the average differences of 10 repeat analyses of the plasma pools LRC 1, LRC 2 and LRC 3 and the target values at three different times of analysis. The GLC values for both total cholesterol and total triacylglycerols are about 5—10% lower than those obtained by the AutoAnalyzer method for the same samples. The bias (mg%) defined as the average of the errors for each pool, varies from 5—17 mg% in direct proportion to the total lipid level. The results have been compared by calculating the relative error in percentage units.

Fig. 5 shows a plot of the values obtained for total cholesterol on 197 plasma samples selected for maximum range of values by the AutoAnalyzer and the automated GLC procedures over a period of a few months. Although in most instances only a single GLC determination was made, an excellent agreement appears to have been realized over the entire concentration range, with a correlation coefficient of 0.9799 and a regression coefficient of 1.0177. The means and standard deviations of the AutoAnalyzer and GLC estimates were 236.28±92.46 and 236.23±96.03 mg%, respectively. The coefficient

TABLE V

COMPARISON OF RESULTS OBTAINED BY AA11 AND GLC METHODS FOR PLASMA POOLS OF WIDELY DIFFERENT TOTAL LIPID CONTENT

Analyses 1—3 were obtained on three separate days within a 2-week period.

Statistic	Target values					
	Cholesterol			Triacylglycerols		
	185	275	396	82	160	290
Analysis 1						
Bias (mg%)	−5	−15	−17	−7	−6	−14
Rel. error (%)	3.0	3.5	5.0	9.0	4.0	5.0
Ave. rel. bias (%)		4.5			6.0	
Analysis 2						
Bias (mg%)	−9	−12	−17	−3	−8	−14
Rel. error (%)	4.8	4.3	5.0	3.6	5.0	5.0
Rel. bias (%)		4.7			4.5	
Analysis 3						
Bias (mg%)	−6	−1	−18	−8	−6	−13
Rel. error (%)	3.2	0.4	4.5	9.7	3.7	4.5
Rel. bias (%)		2.7			6.0	

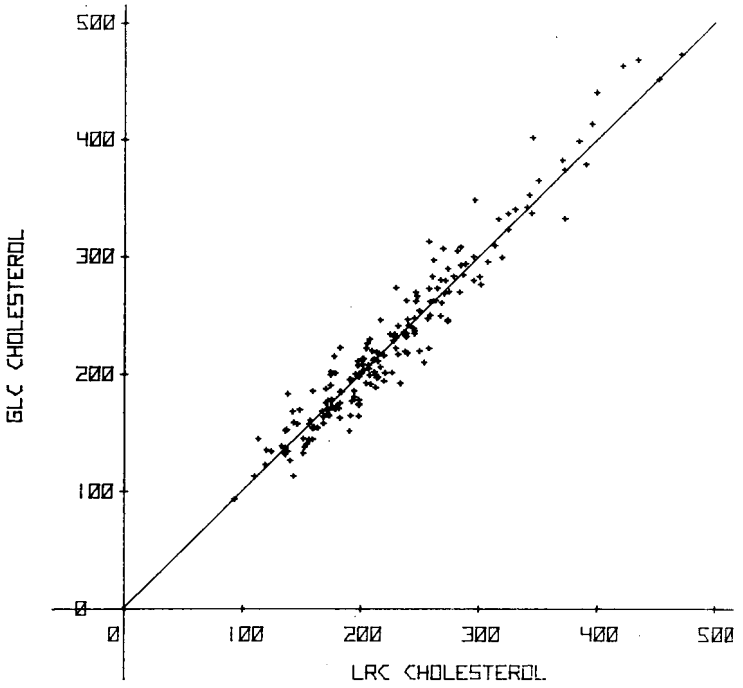

Fig. 5. Comparison of results (mg%) obtained for total plasma cholesterol by GLC and AutoAnalyzer methods over a period of a few months.

of variation between pairs analyzed by the two methods was 4.5%. This indicates that the agreement between the AutoAnalyzer and the automated GLC procedures is about as good as that obtained for repeat analyses of the same sample of plasma by the GLC method alone. Fig. 6 shows a plot of the values obtained for total triacylglycerols on the 197 selected plasma samples. Again with the exception of a few instances only a single determination was made on each sample. The overall correlation coefficient was 0.9837 and the regression coefficient 0.9067. The means and standard deviations of the AutoAnalyzer and the GLC estimates were 345.98±474.08 and 325.16±436.97 mg%, respectively. The extremely high standard deviations arise from the wide range of triacylglycerol concentrations encountered in these samples. The coefficient of variation between duplicates was 8.5%. In contrast to the excellent agreement between the estimates for total cholesterol the AutoAnalyzer and the GLC methods appear to disagree on the values for total plasma triacylglycerols. The GLC method seems to underestimate the total plasma triacylglycerol level by about 20 mg%. An inspection of the plot reveals that there are several plasma samples which differ by up to 50 mg% in the estimated content of triacylglycerols. An examination of the GLC elution patterns and computer print-outs for errors in computation failed to reveal any and suggested that a true bias existed in either of the two methods of analysis. The excellent correlation between the AutoAnalyzer and the GLC estimates, however, indicates that both methods are assessing essentially the same components. It is suggested in discussion that the AutoAnalyzer method may

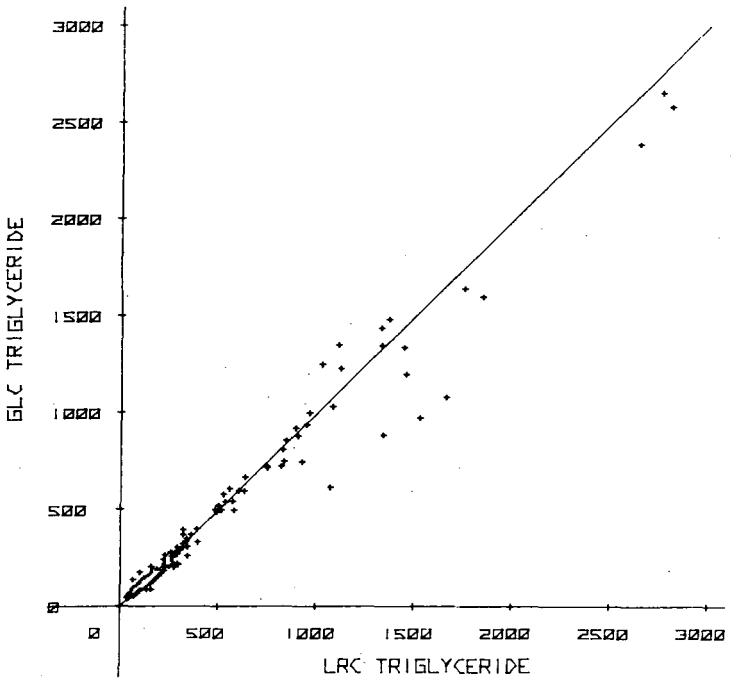

Fig. 6. Comparison of results (mg%) for total plasma triacylglycerols as obtained by GLC and AutoAnalyzer methods over a period of a few months.

give overestimated values due to the manner of expression of data, and due to the presence of non-specific chromogens and partial acylglycerols in the plasma samples.

Fig. 7 shows a plot of the GLC values of total cholesterol versus the AutoAnalyzer target values for 794 random samples of plasma as obtained over a period of two years. Since this collection of samples includes a high proportion of runs recorded using the lower rate of temperature programming there were numerous errors in baseline resetting, the correction of which required the recalculation of the GLC data by cutting out and weighing the peak areas. About 200 samples were corrected in this way. The overall correlation coefficient was 0.98108 with a regression coefficient of 0.9696. The means and standard deviations for total cholesterol of the AutoAnalyzer and the GLC estimates were 217.39±45.93 and 207.03±48.89 mg%, respectively. The coefficient of variation between duplicates was 6.1%. It is seen that over the extended period of time of analysis, involving numerous changes in the operating conditions, columns and liquid phases, as well as recalibrations of the system and dilutions of new batches of internal standard, a good general agreement was realized for the estimates of total plasma cholesterol by the AutoAnalyzer and the automated GLC methods. On the average the GLC method underestimated the total plasma cholesterol by about 10 mg%, while the other parameters of the data remained very much the same as those observed for the short-term correlations.

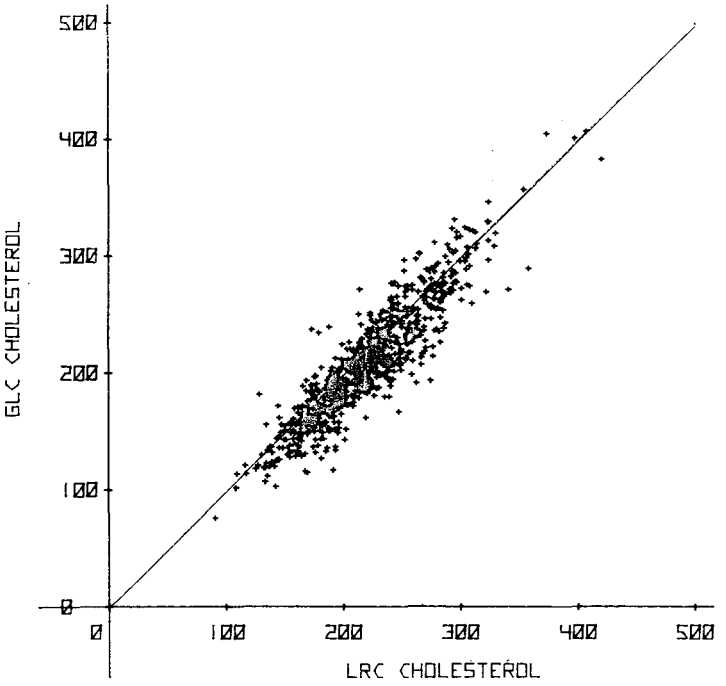

Fig. 7. Comparison of results (mg%) for total plasma cholesterol as obtained by GLC and AutoAnalyzer methods over a period of several years.

Fig. 8 shows a plot of the values for the triacylglycerols as obtained by the AutoAnalyzer and the GLC methods on the 794 random samples of plasma analyzed over a 2-year period. The overall correlation coefficient was 0.9739 with a regression coefficient of 0.9084. The means and standard deviations of the AutoAnalyzer and the GLC estimates were 157.1436±120.9516 and 138.7833±112.8181, respectively. The coefficient of variation between duplicates was 12.7109. It is seen that in the larger number of samples analyzed over the longer period of time, the discrepancy between the GLC and the AutoAnalyzer methods remained about the same as that seen in the smaller number of samples analyzed over the shorter periods of time. In comparison to the AutoAnalyzer, the GLC method underestimated the total plasma triacylglycerols by about 20 mg%.

DISCUSSION

The present large-scale study confirms the general suitability of the automated high-temperature GLC procedure for the analysis of plasma total cholesterol and triacylglycerols claimed previously from analyses of model mixtures of neutral lipids and reference sera. There is evidence that under carefully controlled conditions the plasma lipids can be subjected to a preliminary dephosphorylation with phospholipase C without affecting the estimates for

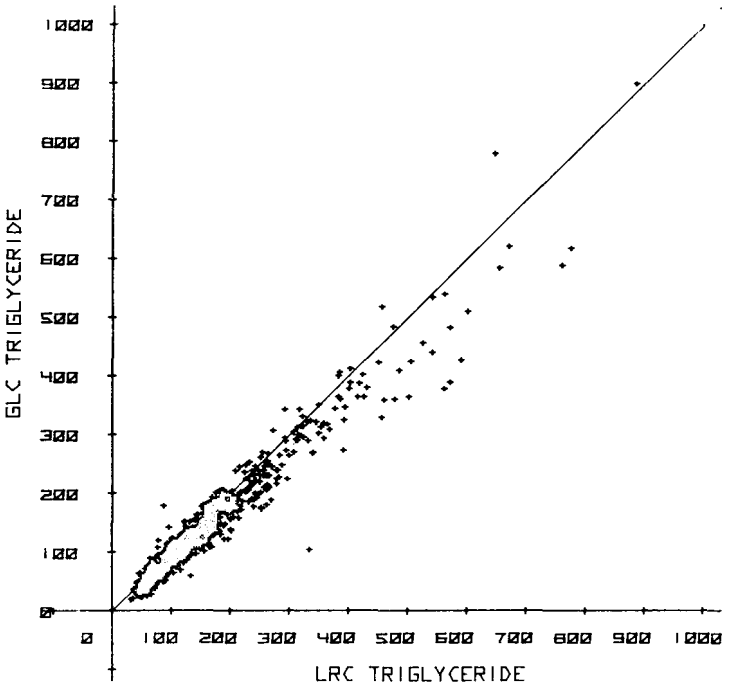

Fig. 8. Comparison of results (mg%) for total plasma triacylglycerols as obtained by GLC and AutoAnalyzer methods over a period of several years.

free cholesterol, cholesteryl esters and triacylglycerols in the digestion residue. We have shown elsewhere [3] that the monoacylglycerols, diacylglycerols and ceramides released by the enzyme treatment yield valid estimates of the plasma lysophosphatidylcholines, phosphatidylcholines and sphingomyelins.

The GLC values obtained for both total cholesterol and total triacylglycerols show excellent correlation with the modified AutoAnalyzer target values, but exhibit a negative bias. Thus, the mass values for total cholesterol are about 5—10 mg% below those of the colorimetric values of the AutoAnalyzer even though a plasma correction has been already made on the data [8, 9]. It is known that the Liebermann-Burchard method gives as much as 20 mg% higher color yield for cholesteryl esters than for free cholesterol and that there are differences in the color yield of different cholesteryl esters [12]. Furthermore, metabolites of cholesterol found in plasma in variable amounts [13] are believed to be responsible for a discrepancy of about 12% between the ferric chloride—sulfuric acid and the GLC or enzymatic methods of analysis of plasma cholesterol in the free form [14]. Since the correction factors applied in the LRC AutoAnalyzer method probably apply only to a narrow range of free cholesterol—cholesteryl ester ratios and to a specific fatty acid composition of the cholesteryl ester, as well as to a specific ratio of cholesterol and its companion sterols, a complete agreement would not be expected between any indirect and direct methods of analysis. It may be noted, however, that a gas chromatography—mass spectrometry (GC—MS) examination

[15] of the plasma free sterol fraction has failed to reveal the high proportions of cholesterol companions previously believed to be present in plasma [13].

A significantly higher intercept value for the AutoAnalyzer method of cholesterol determination, when compared to the GLC method, has also been reported by Watts et al. [5], who, however, did not employ a plasma correction factor. Since they were able to obtain essentially identical values for total cholesterol by both AutoAnalyzer and the GLC methods, when the analyses were made on isolated plasma lipoproteins, it must be concluded that a chromogen is possibly present in the infranatant fraction of plasma lipoproteins obtained after ultracentrifugation at density 1.21 g/ml. This possibility deserves experimental examination.

There is evidence also for plasma interference with the triacylglycerol determination by the AutoAnalyzer method. An intercept value of about 20 mg% obtained in the present experiments compares to an intercept value of 0.58 mmoles/l or about 50 mg% reported by Watts et al. [5] for their AutoAnalyzer—high-temperature GLC comparison. In contrast to the cholesterol determination, the interference for triacylglycerol determination remained relatively constant with the sample concentration, the slope being about 1.0. An examination of the detailed methodology employed by the AutoAnalyzer method reveals that the values are not routinely corrected for a cold alkali blank estimated to be about 3 mg% trioleoylglycerol for the AA11 at Toronto, or for the presence of about 2% mono- and 4% diacylglycerols [16], which could have contributed a maximum of 6—8 mg%, when measured as triacylglycerols. The possibility of occasional contamination of the plasma neutral with polar lipids during the Zeolite adsorption [11] could also have contributed to the higher estimates for total triacylglycerols, as could have the presence of other unidentified components containing actual or potential vicinal hydroxyl groups [8, 11]. Furthermore, the AutoAnalyzer method [8] expresses its results in terms of a C_{54} triacylglycerol or trioleoylglycerol (triolein), which results in a variable overestimation of the content of plasma triacylglycerols averaging 5—8 mg%, since the actual average plasma triacylglycerol is usually a C_{52} [17, 18] or as low as a C_{51} species [18]. Watts et al. [5] reported an average carbon number of 51.8 for the triacylglycerols of normolipemic and hyperlipemic human plasma. The above discrepancies could add up to about 20 mg%, which is the approximate difference observed between the GLC and the AutoAnalyzer methods. Additional discrepancy might arise due to a lack of a hot alkali blank, which for technical reasons also remains uncorrected for in the AutoAnalyzer method. These explanations would account for the discrepancies in the measurements of the unknown plasma samples, as well as would rationalize the lack of disagreement in the measurement of standard trioleoylglycerol from the Lipid Standardization Laboratory, and possibly the smaller differences observed between the GLC and the AutoAnalyzer estimates for triacylglycerols in plasma lipoprotein fractions [5]. The much higher intercept values reported by Watts et al. [5] for the AutoAnalyzer and GLC comparison of plasma total triacylglycerols would require a greater allowance for the above potential interferences or some other basis would have to be found for explanation (subliminal losses of triacylglycerols on GLC during isothermal analysis?). In any event, the present

difference between the GLC and the AutoAnalyzer values is of the order that could be reasonably expected from an examination of the two analytical routines and a knowledge of the plasma lipid composition from independent analyses [16]. On the basis of the above data and the data of Watts et al. [5], it would be desireable to re-examine the AutoAnalyzer methodology of triacylglycerol determination to see if a plasma or serum standardization similar to that employed for cholesterol determination may not be necessary. Certainly the erroneous expression of the final results and the inclusion of the appropriate blanks should be reconsidered for accurate triacylglycerol analyses.

Finally, it should be remembered that the GLC method also could be occasionally in error due to a rapid peroxidation and loss of the more unsaturated glyceryl and cholesteryl esters during isolation, dephosphorylation, derivatization and storage of the plasma lipid samples. Likewise, incomplete dissolution of the plasma lipoproteins from the frozen samples especially after prolonged storage [7] may have contributed more to an underestimation of both total cholesterol and total triacylglycerols than presently appreciated.

The high precision and apparent high accuracy of the present GLC results supports earlier claims in this regard and would seem to recommend it as the method of choice for accurate determination of plasma cholesterol and triacylglycerols. In the present state of development the automated GLC method is capable of determining total cholesterol and total triacylglycerols on a maximum of four samples per hour, which, even when extrapolated to a maximum of 96 samples per day, would not approach the through-put of an AutoAnalyzer (about 300 samples per day). The advantage of the GLC method of plasma lipid determination lies in the definitive nature of the measured components, which is especially important in the analysis of abnormal plasma samples, and in the additional information provided about the composition of the plasma lipids. The separate values for free fatty acids, lysophosphatidylcholines, free and esterified cholesterol, the phosphatidylcholines and sphingomyelins, along with the major molecular species of the cholesteryl esters and the acylglycerols and ceramides are of interest to a variety of clinical conditions and the normal metabolic state of the body.

Obviously, with precise peak area integration the GLC method can provide estimates approaching those sought for plasma cholesterol [19] and plasma triacylglycerols [20] by the absolute or definitive methods of quantitation using stable isotope dilution and combined GC—MS.

ACKNOWLEDGEMENTS

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CHROMBIO. 210

MASS-SPECTROMETRIC STUDY OF THE TRANSESTERIFICATION OF O,N$^{\alpha}$-BIS(TRIFLUOROACETYL) *n*-BUTYL ESTERS OF SOME HYDROXYLATED AMINO ACIDS INTO THEIR O-CARBETHOXY, N$^{\alpha}$-TRIFLUOROACETYL, *n*-BUTYL ESTER DERIVATIVES DURING THE N$^{\tau}$-CARBETHOXY DERIVATIZATION OF HISTIDINE

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SUMMARY

The transesterification of O-TFA, N$^{\alpha}$-TFA, *n*-butyl ester derivatives of some hydroxylated amino acids was studied by gas—liquid chromatography and combined gas—liquid chromatography—mass spectrometry. Changes in elution patterns and fragmentation of the two different O-derivatives are discussed.

INTRODUCTION

In 1974, Moodie [1] successfully resolved the problem of the gas—liquid chromatography (GLC) of histidine by derivatizing the N$^{\tau}$ [2] of the imidazole ring of histidine with ethoxyformic anhydride (EFA) and according to the same paper the GLC peak of tyrosyl-O,N$^{\alpha}$-bis(trifluoroacetyl) *n*-butyl ester (O-TFA,TAB) showed a remarkable diminution.

In our laboratory, the same phenomenon was observed not only for tyrosine but also for some other hydroxylated amino acids during the derivatization of histidine with EFA. In this paper we report investigations on these reactions undergone by four hydroxylated amino acids (threonine, serine, hydroxyproline and tyrosine) using GLC and combined gas—liquid chromatography—mass spectrometry (GLC—MS).

EXPERIMENTAL

Preparation of O-trifluoroacetyl, TAB derivatives (O-TFA,TAB)

The derivatization was carried out according to the procedures developed by Gehrke and co-workers [3—5] and adapted in our laboratory [6]. Into a 10-ml PTFE-lined screw-capped tube was delivered 50 μl of a 10 m*M* solution in 0.1 *N* HCl of threonine, serine, hydroxyproline and tyrosine with N^{ϵ}-monomethyllysine (MML) as internal standard. After evaporation to dryness under nitrogen at 70°, 1 ml of anhydrous *n*-butanol (Merck, Darmstadt, G.F.R.) was added, through which a stream of dry hydrogen chloride was bubbled according to the procedure already described [6].

The esterification was performed for 20 min in a sand-bath maintained at 110°. After the mixture was brought to dryness under a nitrogen stream at 70°, 400 μl of a mixture of trifluoroacetic anhydride (Merck) and dichloromethane (Merck) (1:9, v/v) were added and acylation was carried out at 110° for 60 min after tightening the screw-cap securely.

Preparation of O-carbethoxy, TAB derivatives (O-CEO,TAB)

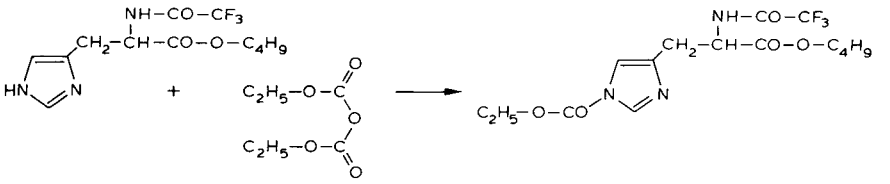
EFA derivatization was then performed immediately. The O-TFA,TAB derivatives obtained were dried under a nitrogen stream at room temperature, showing a drop of temperature due to the volatility of the solution. Then 400 μl of a mixture of EFA (Bayer, Leverkusen, G.F.R.) and dichloromethane (1:500, v/v) was added to the tube, which, after tightening the screw-cap, was heated at 125° for 60 min.

GLC and GLC—MS analysis

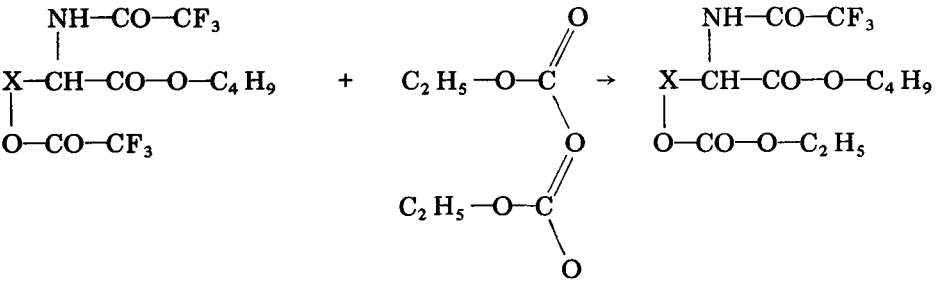
Gas-phase analysis of all derivatives was performed immediately. GLC analyses were carried out with a Packard model 419 gas chromatograph equipped with flame ionization detectors on a 3 m × 3 mm I.D. glass column packed with 1% OV-17 impregnated on Supelcoport. Flow-rates of hydrogen, air and nitrogen were 25, 250 and 30 ml/min, respectively. Temperatures were 205° at the injection port and 225° at the detector. The column temperature was initially held at 85° for 5 min and programmed at a rate of 2°/min. GLC—MS analysis was carried out with an LKB model 9000 gas chromatograph—mass spectrometer. Helium was used as the carrier gas and all the mass spectra were obtained at 28 eV. Other operating parameters were: injection port at 210°, molecular separator at 280°, ion source at 290°, accelerating voltage 3.5 kV, and trap current 60 μA. Mass spectra were recorded by an oscillograph recorder. L-amino acids or DL-amino acids were purchased from Calbiochem and used as received.

RESULTS AND DISCUSSION

In the case of TAB, histidine the underivatized form of imidazole NH is preponderant [1], therefore the reaction with EFA may written as

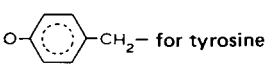


Therefore, the transesterification of O-TFA,TAB to O-CEO,TAB should correspond to the reaction scheme



O—TFA,TAB EFA O—CEO,TAB

where X— is CH_2— for serine, CH_3—CH— for threonine and



For hydroxyproline, the transesterification occurs in the same way.

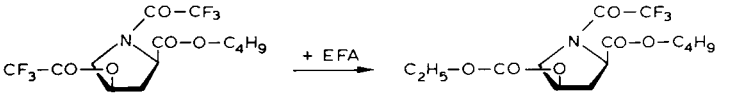


Fig. 1a shows the elution pattern of all amino acids as TAB derivatives when histidine was not eluted. Fig. 1b shows the elution of the same TAB derivatives after reaction with the EFA reagent. Four new peaks appeared (A, B, C, D) together with histidine. These peaks have been identified by mass spectrometry as O-CEO,TAB derivatives of, respectively, threonine, serine, hydroxyproline and tyrosine. When EFA derivatization of amino-acid TAB derivatives was performed with old reagent, the histidine peak was always half that when derivatization was carried out with fresh reagent.

These four amino acids were then studied together as shown in Fig. 2. Fig. 2a gives the elution pattern of O-TFA,TAB derivatives. The peaks in the chromatogram in Fig. 2b were again identified by GLC—MS, as given in Figs. 3—6, and also by comparing the retention times of individually derivatized and chromatographed amino acids. After transesterification to O-CEO, TAB, the retention times were systematically increased by about 12 min, which is equivalent to an increase of about 24° in elution temperature with respect

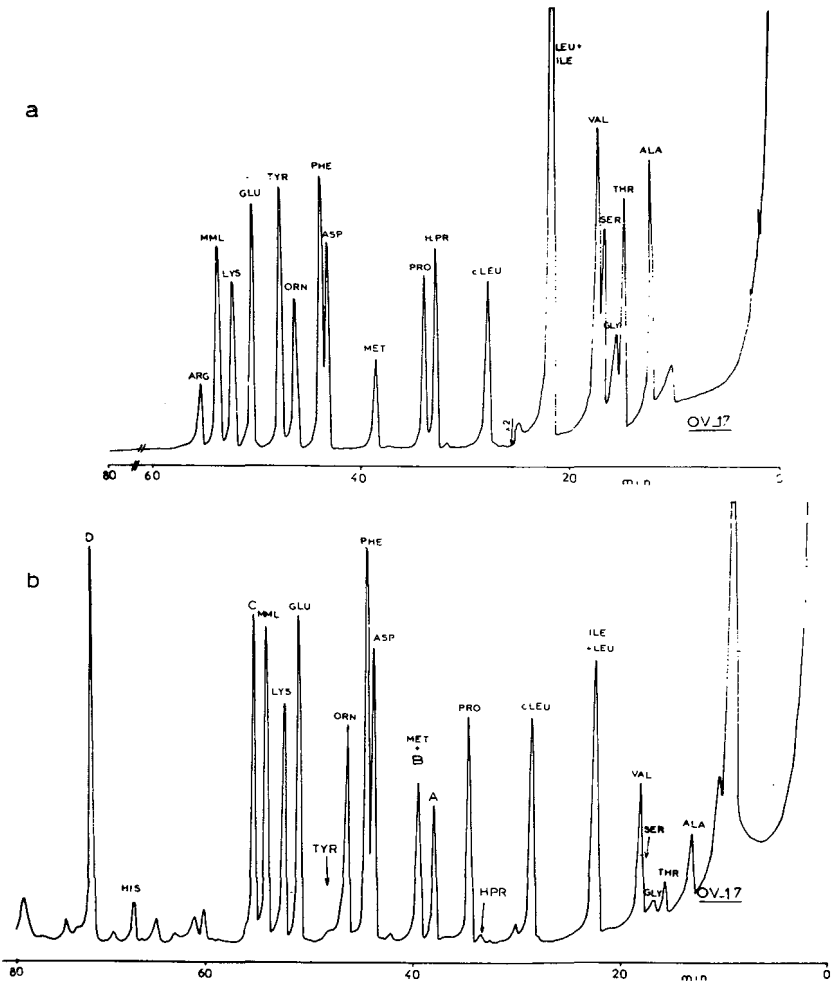


Fig. 1. GLC of 20 reference amino acids on an OV-17 column (cLeu, cycloleucine; MML, N^{ϵ}, monomethyllysine). (a) Chromatogram of the O-TFA,TAB amino-acid derivatives. (b) Chromatogram of the same derivatives treated by a three-week-old EFA mixture.

to that of O-TFA derivatives (Fig. 2a and b and Table I).

As can be seen in Table II, the transesterification yield was in the order tyrosine > serine > hydroxyproline > threonine.

Darbre and Blau [7] investigated the stability of O-TFA amino-acid derivatives by following their progressive hydrolysis in methylethyl ketone containing 5% water. Our results coincide with their hydrolysis order. It can therefore be said that the transesterification relates to ease of hydrolysis.

The fragmentations of O-TFA,TAB and O-CEO,TAB derivatives are shown in Table III and can be interpreted as follows.

(1) For the fragment M—101 there is a difference of 24 a.m.u. which is due to the difference of mass between O-TFA and O-CEO derivatives.

(2) In the case of serine and threonine, we have observed some common fragments and some other specific fragments for the O-CEO compounds.

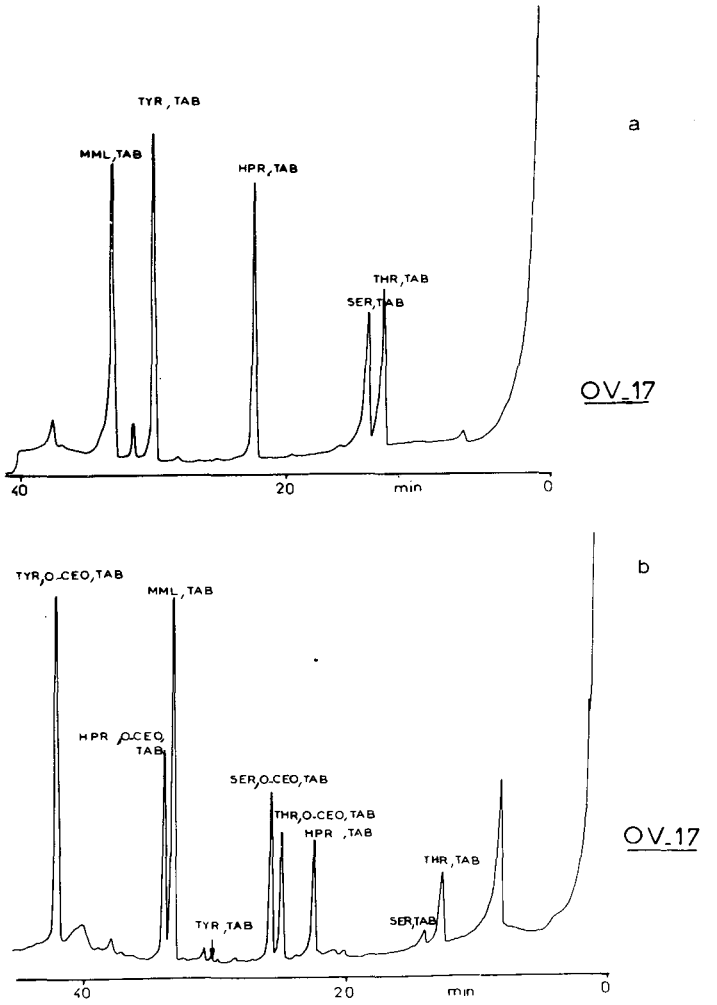


Fig. 2. Transesterification of four hydrolated L-amino acids: serine, threonine, hydroxyproline and tyrosine derivatives. (a) Chromatogram of the four amino acids in the form of O-TFA,TAB. (b) Chromatogram of the same derivatized amino acids after reaction with EFA. The new four peaks were identified as threonine O-CEO,TAB, serine O-CEO,TAB, hydroxyproline O-CEO,TAB and tyrosine O-CEO,TAB.

Both hydroxyproline O-TFA,TAB and O-CEO,TAB give the same fragments by eliminating $COOC_4H_9$ or $O—CO—CF_3$ and $O—COOC_2H_5$, respectively. The fact that tyrosine derivatives do not have fragments in common at relatively high mass ($m/e > 200$) can be explained by preferential fragmentation in the hydrocarbon side chain of the benzene ring.

(3) Fragment 113, lost during electron-impact ionization of O-TFA,TAB derivatives, has two possible structures: $NH_2—CO—CF_3$ or $O—CO—CF_3$. But with O-CEO,TAB derivatives the deleted fragment has $m/e = 89$, corresponding to $O—COO—C_2H_5$. Thus we may assign the $O—CO—CF_3$ structure to the fragment 113 of the O—TFA,TAB derivatives. Consequently we dem-

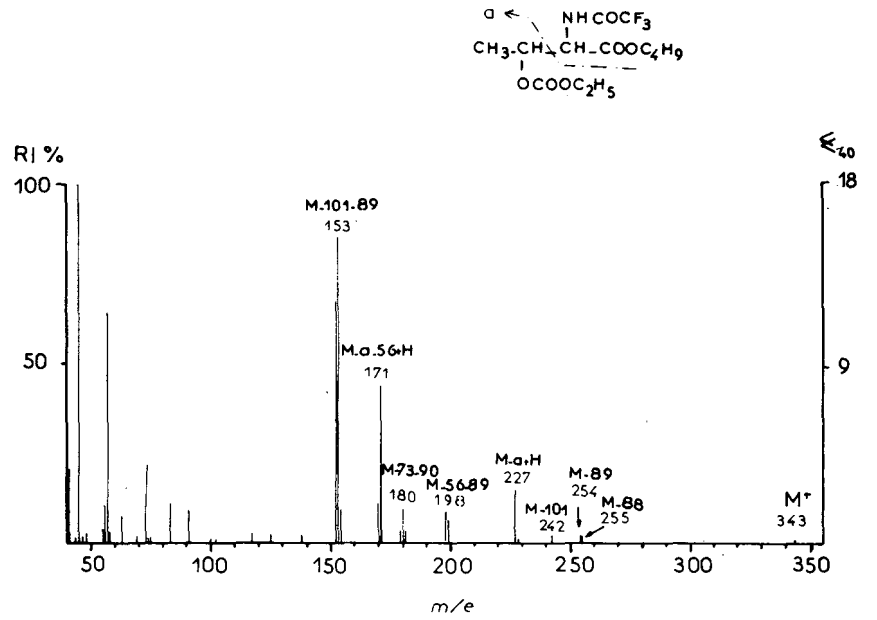


Fig. 3. Mass spectrum of threonine O-CEO, TAB derivative. See text for experimental conditions.

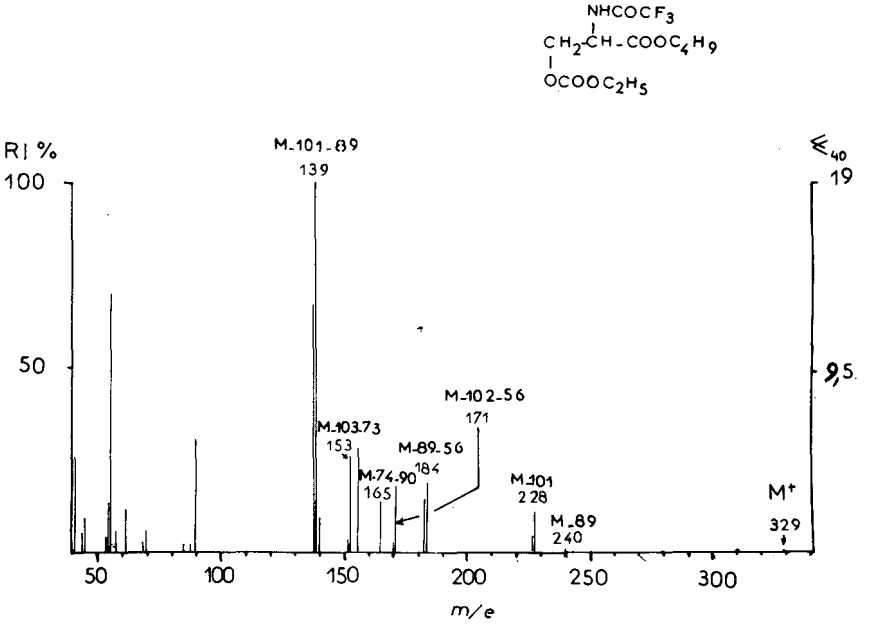


Fig. 4. Mass spectrum of serine O-CEO,TAB derivative, as in Fig. 3.

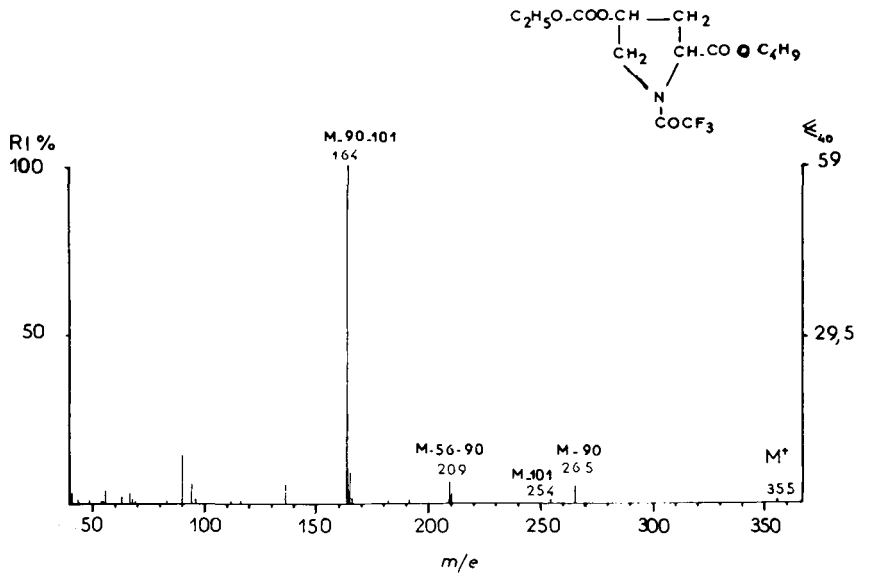


Fig. 5. Mass spectrum of hydroxyproline O-CEO,TAB derivatives, as in Fig. 3.

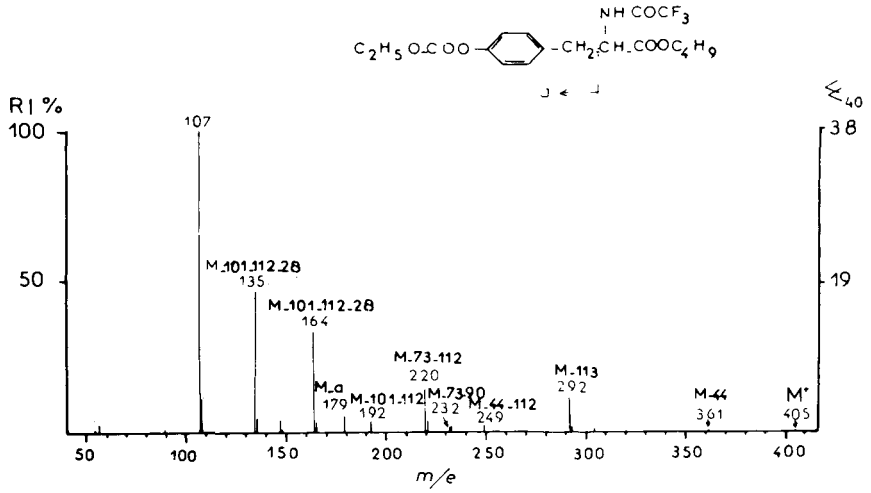


Fig. 6. Mass spectrum of tyrosine O-CEO,TAB derivatives, as in Fig. 3.

onstrate a fragmentation which corroborates well the fragmentation mechanism proposed by Gelpi et al. [8] and recently by Padieu et al. [9]. During electron-impact ionization the bond between alcoholic oxygen and the aliphatic hydrocarbon chain is more labile than that between the NH—CO—CF_3 group and the amino acid C^{α}, whereas the bond between the benzene ring of tyrosine and the phenolic oxygen is more stable than that between the NH—CO—CF_3 group and C^{α}.

TABLE I

RELATIVE RETENTION TIMES OF O-TFA,TAB AND O-CEO,TAB DERIVATIVES OF THE FOUR HYDROXYLATED AMINO ACIDS

Values with respect to N^{ϵ}-monomethyllysine as zero-time reference.

	Retention time (min)		Increase in retention time (min)
	O-TFA,TAB	O-CEO,TAB	
Threonine	−21	−8	13
Serine	−20	−8	12
Hydroxyproline	−11	+1	12
Tyrosine	−3	+9	12

TABLE II

TRANSESTERIFICATION FOR THE RELATIVE LEVELS OF THE FOUR HYDROXYLATED AMINO ACIDS

Values calculated as follows: in columns 1, 2 and 3, response coefficient R_c = amino acid peak height/MML peak height; in column 4, percentage transesterification = (value column 1 − value column2)/value column 1.

	Before reaction with EFA	After reaction with EFA		Percentage transesterification
	O-TFA,TAB	O-TFA,TAB remaining	O-CEO,TAB	
	(1)	(2)	(3)	(4)
Threonine	0.59	0.19	0.35	68
Serine	0.51	0.04	0.47	92
Hydroxyproline	1.05	0.31	0.58	70
Tyrosine	1.31		0.97	100

CONCLUSION

The transesterification of O-TFA,TAB into O-CEO,TAB, a side-reaction during the N^{τ}-carbethoxy derivatization of histidine and other imidazole compounds such as histamine, has already rendered very much easier the interpretation of mass spectra of some hydroxylated amino acids. Furthermore, this reaction may have a possible use for the determination of free hydroxyl groups in compounds other than amino acids.

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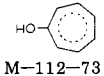
TABLE III

MAIN FRAGMENTS OBTAINED BY ELECTRON-IMPACT IONIZATION GLC—MS

O-TFA,TAB and O-CEO,TAB derivatives of: (a) serine and threonine, (b) tyrosine and hydroxyproline. See text for experimental conditions.

Fragments	Serine O-TFA,TAB M^+ 353		Serine O-CEO,TAB M^+ 329		Fragments	Threonine O-TFA,TAB M^+ 367		Threonine O-CEO,TAB M^+ 343	
	m/e	RI (%)	*m/e*	RI (%)		*m/e*	RI (%)	*m/e*	RI (%)
M−101 M−$COOC_4H_9$	252	1.7	228	10.5	M−101 M−$COOC_4H_9$	266	3.4	242	2.3
M−113−56 M−CF_3COO−C_4H_8	184	2.7			M−140 M−CH_2=$CHOCOCF_3$	227	3.3		
M−89−56 M−C_2H_5OCOO−C_4H_8			184	18.6	M−116 M−CH_2=$CHOCOOC_2H_5$			227	14.8
M−114−74 M−CF_3COOH−C_4H_9OH	165	4.2			M−113−56 M−CF_3COO−C_4H_8	198	4.8		
M−90−74 M−C_2H_5OCOOH−C_4H_9OH			165	12.8	M−89−56 M−C_2H_5OCOO−C_4H_8			198	6.7
M−113−101 M−CF_3COO−$COOC_4H_9$	139	57.3			M−114−73 M−CF_3COOH−C_4H_9O	180	7.6		
M−89−101 M−C_2H_5OCOO−$COOC_4H_9$			139	100	M−90−73 M−C_2H_5OCOOH−C_4H_9O			180	9.6
C_4H_9	57	100	57	3	M−140−56 M−CH_2=$CHOCOCF_3$−C_4H_8	171	12		
M−89 M−C_2H_5OCOO			240	1.8	M−116−56 M−CH_2=$CHOCOOC_2H_5$−C_4H_8			171	45
M−102−56 M−$CHOCOOC_2H_5$−C_4H_8			171	17	M−113−101 M−CF_3COO−$COOC_4H_9$	153	100		
M−103−73 M−$CH_2OCOOC_2H_5$−C_4H_9O			153	25.6	M−89−101 M−C_2H_5OCOO−$COOC_4H_9$			153	85.2
					M−88 M−C_2H_4OCOO			255	1.9
					M−89 M−C_2H_5OCOO			254	1.9
					C_2H_5O			45	100

TABLE III (continued)

Fragments	Tyrosine O-TFA,TAB M⁺ 429		Tyrosine O-carbethoxy,TAB M⁺ 405		Fragments	Hydroxyproline O-TFA,TAB M⁺ 379		Hydroxyproline O-carbethoxy,TAB M⁺ 355	
	m/e	RI (%)	*m/e*	RI (%)		*m/e*	RI (%)	*m/e*	RI (%)
M	429	0.1	405	0.1	M	379	0.1	355	0.04
M–101 M–$COOC_4H_9$	328	9.9	304	1.2	M–101 M–$COOC_4H_9$	278	16.0	254	0.2
M–113 M–NH_2COCF_3	316	34.1	292	11	M–114 M–CF_3COOH	265	2.5		
M–113–73 M–NH_2COCF_3–C_4H_9O	243	14.3			M–90 M–C_2H_5OCOOH			265	4.7
M–112–101 M–$NHCOCF_3$–$COOC_4H_9$	216	6.5	192	3.7	M–114–56 M–CF_3COOH–C_4H_8	209	1.6		
M–226 NHCOCF₃ M–HC–$COOC_4H_9$	203	100	179	6.3	M–90–56 M–C_2H_5OCOOH–C_4H_8			209	5.7
	107	14.3	107	100	M–114–101 M–CF_3COOH–$COOC_4H_9$	164	100		
					M–90–101 M–C_2H_5OCOOH–$COOC_4H_9$			164	100
M–112–73 M–$NHCOCF_3$–C_4H_9O			220	14.6					
M–44 M–C_2H_4O			361	0.3					
M–90–73 M–C_2H_5OCOOH–C_4H_9O			232	2.0					
M–112–44 M–$NHCOCF_3$–C_2H_4O			249	2.6					

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CHROMBIO. 206

AUTOMATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SAMPLE CLEAN-UP FOR MASS FRAGMENTOGRAPHIC ASSAYS

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SUMMARY

A previously described high-performance liquid chromatographic (HPLC) sample clean-up procedure has been automated by attaching a (DuPont) auto-sampler and a time-controlled fraction collector to the HPLC equipment. To obtain the required reliability for unattended operation, the sample intake was controlled by volume rather than by time, and the system was protected against sample loss due to non- or improper operation of the injection valve. The capacity of the system depends on the HPLC run time per sample but varies from 45 to 135 samples per 24 h. The recovery and reproducibility are comparable to the manually operated system, while carry-over to subsequent samples is prevented by intermittent injection of the HPLC solvent system as flush fluid.

INTRODUCTION

In a previous paper [1] the use of HPLC for sample clean-up in mass fragmentographic assays was described. It was demonstrated that the recovery of the compound to be determined and the extent of purification was improved when compared with a clean-up by a back-extraction procedure. Additional advantages of the HPLC procedure mentioned were the increased total analysing capacity of up to an average of e.g. 60 samples a day and the possibilities for automation. The present paper describes the set-up and performance of an automated HPLC clean-up equipment for unattended 24-h operation.

MATERIALS AND METHODS

Equipment

The major components of the automated HPLC system are:

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(i) A DuPont 834 Automatic Sampling System (auto-sampler; DuPont, Wilmington, Del., U.S.A.) equipped with the low-volume sample option and a pneumatically operated 6-port 48 · 10^6 Pa injection valve (Rheodyne, Berkeley, Calif., U.S.A.). The volume of the injection valve loop is 500 μl.

(ii) A Waters Assoc. (Milford, Mass., U.S.A.) Model ALC 202 high-performance liquid chromatograph equipped with a 30 cm × 4 mm I.D. stainless-steel column filled with μPorasil (10 μm; Waters Assoc.). The elution solvent was composed of *n*-hexane—isopropanol (80:20, v/v) to which 4% of ethanol and 0.1% of concentrated ammonia were added. The standard UV detector was operated at 280 nm.

(iii) A Gilson Microcol TDC 80 fraction collector (Gilson, Villiers-le-Bel, France) for 80 tubes, with built-in drop counter.

(iv) A Kipp BD 9 (Kipp, Delft, The Netherlands) two-pen potentiometer recorder with remote control of chart drive and electrical pen lift.

System design

The design of the automated system is schematically illustrated in Fig. 1. The injection valve is connected by 0.25 mm I.D. stainless-steel capillaries to the HPLC pump and column. A drop counter (fraction collector accessory) is connected to the drain outlet of the injection valve.

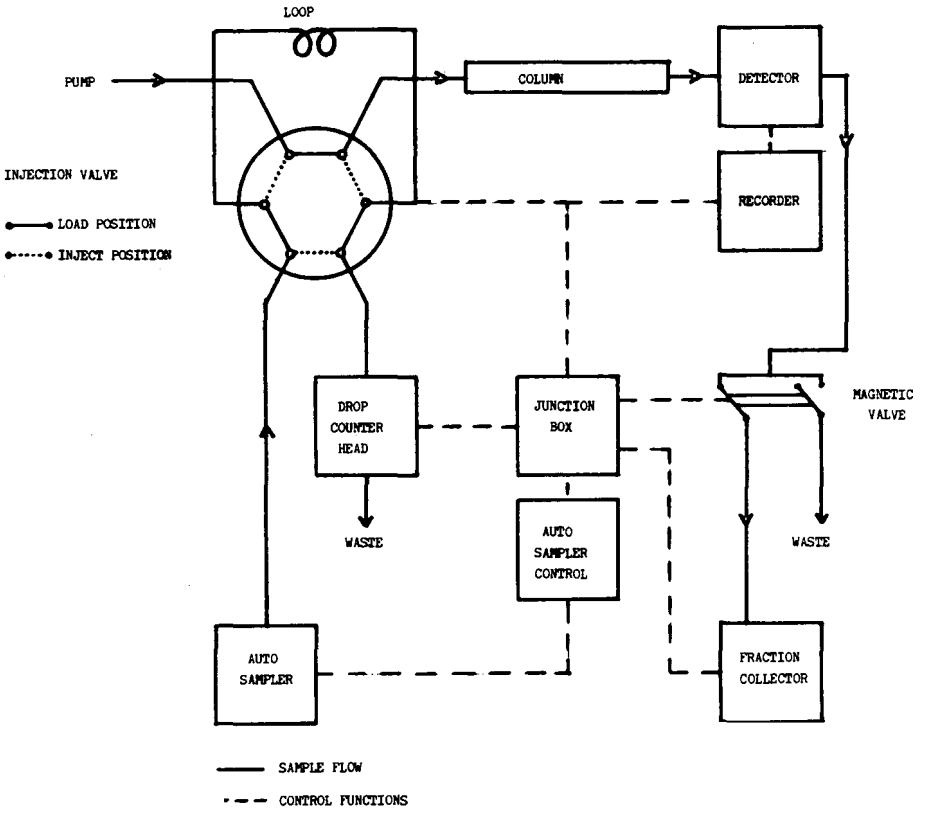

Fig. 1. Design of automated HPLC system for sample clean-up.

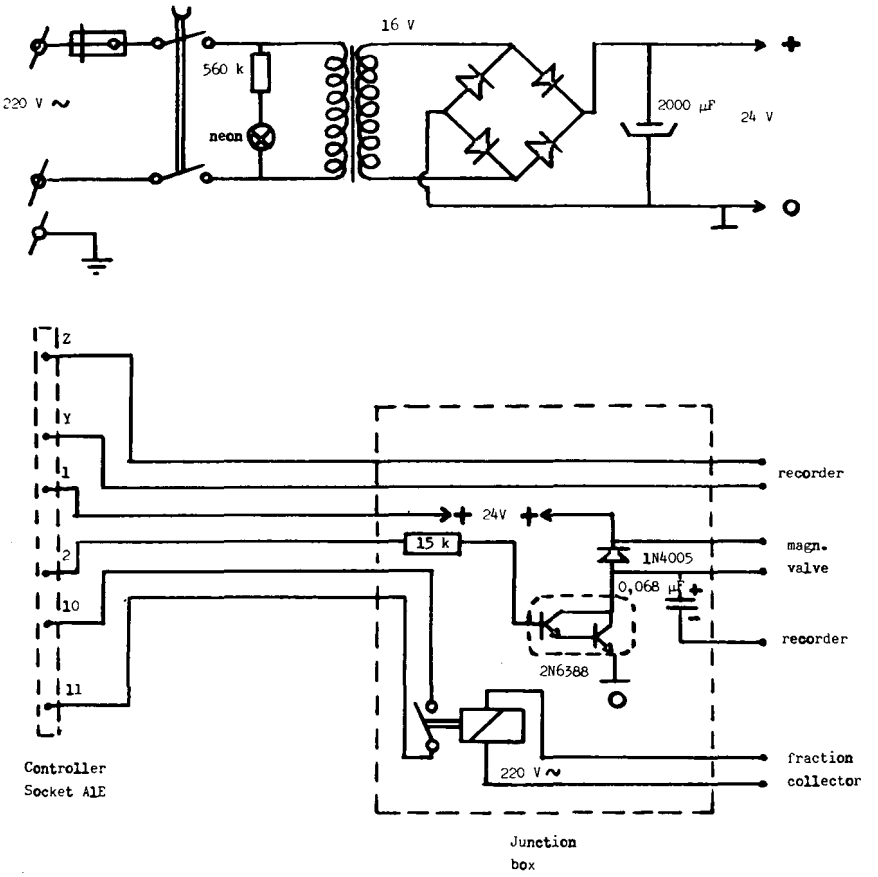

Fig. 2. Schematic representation of junction box and interconnections between the components of the automated system.

The column outlet is fed to a T-piece of which the two other sides are connected by flexible 0.5 mm I.D. tubing to the fraction collector and waste flask. A magnetically operated valve (under control of the auto-sampler timer) blocks either of those two routes and effectively directs the eluate flow either to the sample collection vial or to the waste.

The interconnections between the auto-sampler, magnetic valve, fraction collector and recorder are made via a home made junction box of which the electronic circuit is shown in Fig. 2.

The main power supply of the HPLC-pump is switched by a 24-h timer to enable pump switch-off at a predetermined time after having processed the last sample.

In general, HPLC and also gas chromatographic (GC) auto-samplers are used to inject a small volume, e.g. 2 µl from a larger sample volume, e.g. 2 ml. For the present preparative purpose, however, as much as possible of the sample should be injected, while the maximum injected volume is determined by the minimum required chromatographic performance. Obviously, the relative loss of sample as residue in the sample vial, in the injection needle and

in the capillary to the injection valve is smaller for greater sample volumes. Injection of volumes greater than 0.5 ml, however, might impair the chromatographic performance.

To minimize losses by residual sample, the sample vials used were of the shape shown in Fig. 3. By injecting no more than 450 µl of a sample of 500 µl, the narrow end tip of the vial will remain filled, preventing air entering the system and causing a 90% sample consumption. Because of the great variability in the dimensions of the DuPont narrow-tipped sample vials, causing not only irreproducible sample losses, but also failures in the vial transport, narrow tolerance home-made vials were used.

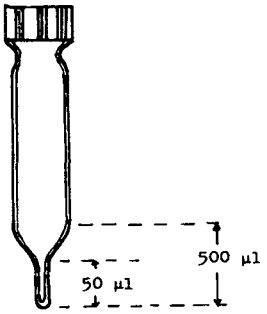

Fig. 3. Screw capped (PTFE rubber laminated septum) sample vial for a DuPont auto-sampler.

Operating procedure

Plasma extracts are evaporated to dryness. The residues are redissolved and transferred into the auto-sampler sample tubes (see Results and discussion for tube specifications) by means of two volumes of 250 µl each of the HPLC elution solvent. When necessary, flush vials (distinguished by their white caps) containing pure elution solvent can be placed intermittently with the black-capped sample vials in the rack.

The drop counter is adjusted to, e.g., 35 drops, corresponding to 0.42—0.45 ml. The auto sampler control timers "start integrate", and "stop integrate" (originally meant to control an auxilliary integrator) are set for the desired start and end time of sample collection. The "total time" controller is set to define the total run time per sample.

After starting the system, the following sequence of operations (illustrated in the time diagram of Fig. 4) is carried out automatically. The first sample vial is brought into position and the tube guiding head of the fraction collector moves to the first tube. The dual injection needle system moves down and pierces through the PTFE rubber laminated septum into the sample vial, down to approximately 1 mm from the bottom of the narrow tip. The vial headspace is pressurized up to $240 \cdot 10^3$ Pa to flush the sample through the injector loop. When the preset number of drops of elution solvent is measured by the drop counter at the injection valve drain outlet, the valve is switched to the inject position, the vial headspace is depressurized and the analysis time counter starts. At the preset "start integrate" time the magnetic

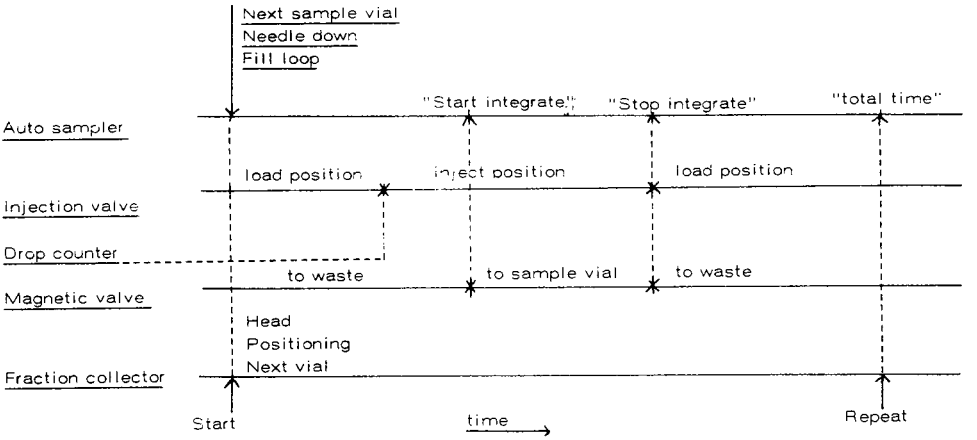

Fig. 4. Time diagram of different system functions for one sequence.

valve opens the column effluent tube to the fraction collector and closes the way to the waste. At "stop integrate" time the magnetic valve is switched again to direct the flow to the waste and blocks the way to the fraction collector tubes. At the same time, the injection valve is reset to the load position.

At the preset "total time" the injection needle system is moved upwards, and a new sequence is started. When flush samples are used, the injection valve is not operated and the subsequent sample is brought forward after the preset volume (equal to the sample inject volume) is flushed through the loop.

RESULTS AND DISCUSSION

Reliability

Auto-samplers are introduced for economic and quality reasons. It is expected that they increase the total analyzing capacity and reduce dull and time-consuming work, while their reproducibility may improve assay quality and may reduce mistakes.

A straight forward connection of the standard DuPont auto-sampler to the HPLC-equipment caused a number of problems resulting either in complete loss of samples or caused failures requiring reprocessing of saved samples. Some of these problems are, however, inherent to the specific use for preparative handling of biological samples. Because the advantages of automation are only achieved when the system can be operated unattended, the required modifications are discussed.

In the normal operating procedure for the DuPont auto-sampler, the injection loop is not filled by controlling the volume of fluid at the drain outlet, but by pressurizing the head space of the sample vials for a preset time. This procedure, however, cannot be used for routine analyses of biological samples. In normal practice, the flow resistance in the line from injection needle to injection valve drain outlet, as well as the head-space pressure will vary from day to day, or even from sample to sample. This variability causes irreproducible volumes of injected sample. The extremes are that, at low resistance and/or

high pressure, the sample vials are emptied and air enters the system whereas at high resistance and/or low pressure the valve is switched to the inject position before the loop was adequately filled. Pressure variations may be due to leaking vial septa or sample pump solenoid valve and to a minor extent to pressure variations in the air supply. Flow resistance variations are caused by deposits in the capillary and injection valve as a result of the use of relatively crude extracts of biological material.

Measuring the injection volume, instead of operating in the time-controlled mode circumvents these problems, although regular cleaning of the system by flushing with appropriate solvents or by injecting alternately sample and rinsing solvent, remains necessary for maintenance of proper system performance.

Owing to normal wear or to accidental failure in the pneumatic system, the injection valve sometimes fails to operate, and the samples will be lost because the sample vial transport and sample intake systems will continue operation. The system can be safeguarded against this fatal failure by mounting a micro-switch under the injection valve, which switches off the auto-sampler when the injection valve does not rotate while the analysing time-counter is running.

Failures in the auto-sampler vial transport and needle injection system will only result in loss of capacity while saving the samples, as long as no injections were made. When the injections were made at incorrect times (which sometimes occurs with our auto sampler, probably due to electronic failures) all samples are lost. Unfortunately, the system cannot be protected against this rarely occurring but fatal failure.

Recovery

The recovery of the automated clean-up procedure in daily routine operation has been determined by processing blank plasma extract spiked with tritiated mianserin [2] (20 nCi, 50 ng per injection) and measuring the amount of radioactivity in the vials of the fraction collector. In this set-up, the recovery was proved to be (67.4 ± 2.8)% (mean ± S.D., n = 17). The maximum possible recovery is determined by the injection and sample collection efficiency. For the manually operated system, it has already been shown [1] that the recovery of injected material is 100%. Any reduction of recovery should therefore come from the injection side. As a minimum of 30—50 μl of the sample solution should remain in the vial tip and because of the total capillary volume from needle to injection valve of 30 μl, the minimum loss of sample equals 80 μl. With a sample and loop volume of 500 μl the maximal recovery therefore equals approx. 85%. Although this would be sufficient for most applications, the recovery could be improved by using a 1 or 1.5 ml sample and loop volume.

Only in case the retention time of the compound to be analysed is shifted during sample processing, the recovery might be reduced because the compound might no longer be completely eluted within the predetermined fraction-collection period.

Retention time variations which are not notified (by shifting retention times of marker compounds) in unattended automated systems, do occur in

daily routine analyses, e.g. by temperature variations. To minimize the risk of collecting the wrong fraction the collecting interval should not be chosen too short or the magnetic valve should be triggered by the appearance of a marker peak.

Carry-over

The carry-over, or contribution to the next sample was determined to be less than 0.1% for the manually operated system. When the automated system is operated without vials containing flush solution, the carry-over is determined by the contents of the injection needle and PTFE capillary to the injection valve. As this volume is 30 μl, the minimum carry-over to the next sample is 6% for a sample of 500 μl. For samples of more or less the same concentration this might be acceptable. In case of a greater concentration variability, however, this carry-over will yield unacceptable inaccuracies and flush solution vials must be used.

Reproducibility

When dealing with internal standards, the reproducibility of the injection is not critical. In case no internal standard is used, the overall assay reproducibility is also determined by the injection reproducibility. Because the reproducibility of the injected volume is ±1 drop, the use of a narrow bore capillary results in small droplets of approx. 15 μl at the drain outlet, corresponding to an injection reproducibility of ± 3.5% at an injected sample volume of 420 μl. This injection reproducibility is in good agreement with the overall reproducibility of 4.2% found in the recovery experiments in a set-up of daily routine processing of tritiated mianserin (vide supra).

Capacity

The capacity, expressed as number of samples which can be automatically processed per unit time, depends upon the retention time of the compound to be measured and the retention times of co-extracted endogenous material which should be eluted prior to the injection of the next sample.

The capacity is illustrated by two different assay methods. One is the relatively simple and fast analysis of the antidepressant mianserin (Bolvidon®) in human plasma [2]. Because of the relatively clean hexane extract used in this method, one complete HPLC run takes no more than 10 min yielding a capacity of approx. 6 samples per hour. When flush samples are used, one auto-sampler rack can contain 45 real samples which can be processed in 8–9 hours.

For the assay of the anti-arrhythmic amino steroid Org 6001* [3], however, an ethyl acetate extraction is used causing co-extraction of interfering impurities with HPLC retention times of up to 0.5 h. In the manually operated procedure the elution of these impurities can be speeded up by increasing for some minutes the flow-rate from 0.7 ml/min to e.g. 4.7 ml/min after the appropriate eluate fraction has been collected. In the automated procedure the total HPLC run time should be 0.5 h per sample, because the flow-rate

*3α-Amino-2β-hydroxy 5α-androstan-17-one-hydrochloride.

cannot be controlled by the auto-sampler. Although in this case the capacity is only 2 samples per hour, the number of samples which can be processed unattended outside normal working hours (8 h) is 30—35, while the total 24-h day capacity equals 45—50 samples, which is about the working day capacity of the GC—mass spectrometric equipment.

CONCLUSIONS

Attachment of an auto-sampler and a time-controlled fraction collector to a high-performance liquid chromatograph results in an automated system for the clean-up of extracts of biological samples, which can process unattended 45—135 samples per 24 h, depending on the required HPLC retention time.

Intermittent processing of flush samples, automatic volume control of sample intake and provisions against sample loss owing to non- or improper operation of the injection valve enable unattended (e.g. overnight) operation with recoveries and reproducibilities comparable to the manually operated system.

ACKNOWLEDGEMENTS

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Biomedical Applications

CHROMBIO. 208

FLUORIMETRIC DETECTION OF OCTOPAMINE IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ITS APPLICATION TO THE ASSAY OF DOPAMINE β-MONOOXYGENASE IN HUMAN SERUM

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SUMMARY

A high-performance liquid chromatographic procedure is described for the determination of octopamine. The method, which is based on the separation on a microparticulate bonded strong cation-exchange resin and measurement of the native fluorescence, has been applied to give a sensitive assay of dopamine β-monooxygenase (EC 1.14.17.1) activity in human serum with tyramine as the substrate. The procedure, which has been designed for use with an automatic sampler, has a detection limit of about 50 pmoles of octopamine, and the analysis time is approximately 10 min per sample.

INTRODUCTION

A number of high-performance liquid chromatographic (HPLC) systems have recently been found to offer convenient methods for the microanalysis of catecholamines [1—4], using different detectors. So far, three principles of detection have been used: an electrochemical detector [1], a UV detector [2—4] and, more recently, fluorimetric detection following derivatization with *o*-phthalaldehyde [5]. Due to the relatively low sensitivity of the UV detector and the experimental complexity of the other two methods of detection, an alternative method has been developed in the present study.

The native fluorescence of catecholamines is considered to be weak [6], but has nevertheless been used for a simple and rapid assay of total catecholamines in tissues such as the adrenal gland [7]. The application of this method to crude extracts of biological materials is, however, complicated by considerable interference from various endogenous constituents which exhibit native fluorescence, at the same or similar wavelengths as the catecholamines, or by compounds which quench the catecholamine fluorescence [8]. These problems

are eliminated when HPLC is used. In the present report we describe the detection of octopamine (and tyramine), based on its native fluorescence, which gives a sensitivity comparable to the electrochemical detector [1] and the fluorescence derivative method [5]. However, due to its simpler experimental approach, native fluorescence can also be more easily applied to automated analysis to meet the special requirements of multiple analyses. We have found the method very useful in the assay of the dopamine β-monooxygenase (dopamine β-hydroxylase) catalysed hydroxylation of tyramine to octopamine in, for example, human serum.

EXPERIMENTAL

Materials

Tyramine HCl was obtained from Koch-Light (Colnbrook, Great Britain), DL-octopamine HCl (*p*-hydroxyphenyl ethanolamine) and catalase (crystalline, thymol free) from the Sigma Chemical Co. (St. Louis, U.S.A.), and fusaric acid from ICN, (U.S.A.). All other chemicals were obtained from various commercial sources and were of analytical grade quality.

Dopamine β-monooxygenase was purified from bovine adrenal medulla as described earlier [9].

Serum samples were collected from healthy adults and stored at 0° until used.

HPLC analysis

A constant-volume HPLC pump (Model Constametric II G from Laboratory Data Control, Riviera Beach, Fla., U.S.A.) supplied with a Rheodyne valve-loop injector (20- and 100-μl loops) was used. The chromatographic components were detected using a spectrofluorometer (Model SFM 22 from Kontron, Zürich, Switzerland) supplied with a 150-W Xenon lamp (XBO 150 W/1 from Osram, München, G.F.R.) and a 20-μl flow-through cell. The instrument was operated at the highest range of the gain setting ("High HV var"). Fluorescence excitation and emission spectra were monitored by an X-Y recorder (Model 7035 from Hewlett-Packard). No corrections were made for photomultiplier response, monochromator sensitivity or Xenon arc-lamp emission. The fluorescence intensity was expressed on a linear scale.

The chromatographic separation was achieved at 20° on a microparticulate bonded strong cation exchanger (Partisil-10 SCX, pre-packed from Whatman in a 25.9 cm × 4.6 mm I.D. stainless-steel tube) with a theoretical plate number of 21,5000/m. A short pre-column (40 × 2 mm I.D. stainless-steel tube) packed with pellicular silica (HC Pellosil from Whatman) was used to protect the cation-exchange column. The mobile phase, consisting of 50 m*M* acetate buffer (pH 4.2), was pumped at a flow-rate of 2 ml/min (1400 p.s.i.)

Assay of dopamine β-monooxygenase activity

The assay of dopamine β-monooxygenase activity in human serum was performed as described by Fujita et al. [5], but with the following modifications. The pH was 6.0 and the reaction mixture also contained 50 m*M* 2-(N-morpholino)-ethanesulphonic acid (see Results section). The reaction was

quenched by adding an equal volume of ethanol containing 1.0 m*M* fusaric acid, and the mixture was left at 0° for 30 min before centrifugation at 10,000 rpm for 10 min (Eppendorf Model 5412 Microfuge). The supernatant fluid was diluted (4—20 times, depending on the enzymic activity of the serum analysed) with 50 m*M* acetate buffer (pH 4.2), and 20—100-μl aliquots were injected into the liquid chromatograph. The concentrations of the unknown samples were determined from standard curves.

Purified dopamine β-monooxygenase was also assayed as described by Wallace et al. [10].

RESULTS

Fluorescence properties of octopamine

The uncorrected fluorescence excitation and emission spectra of octopamine are shown in Fig. 1. The amine (10 nmoles) was injected into the liquid chromatograph, and when the peak height was reached the solvent flow was stopped and the spectra recorded. It is seen that the solvent contributed very little to the spectra of octopamine (λ_{ex} = 280 nm and λ_{em} = 303 nm). The fluorescence properties of tyramine were very similar to those of octopamine.

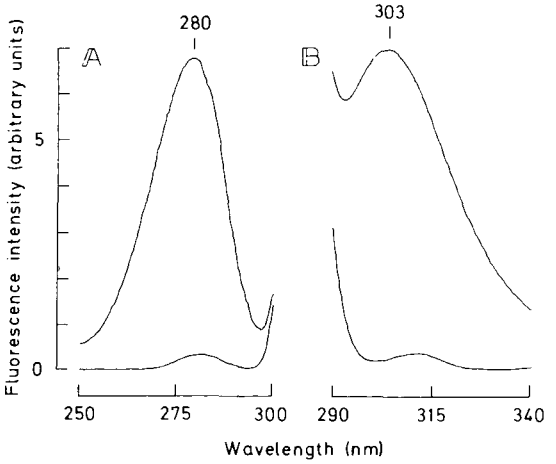

Fig. 1. Uncorrected fluorescence excitation (A) and emission (B) spectra of octopamine at 20° obtained by a Model SFM-22 spectrofluorometer from Kontron, supplied with a 20-μl flow-through cell. The fluorescence spectra of the mobile phase represent the baselines in (A) and (B). λ_{ex} = 280 nm and λ_{em} = 303 nm were selected for the recording of the fluorescence emission and excitation spectra, respectively. For experimental details, see text.

Chromatographic conditions

A microparticulate bonded strong cation-exchange resin was selected for the separation of octopamine from tyramine using a 50 m*M* acetate buffer, (pH 4.2) as the mobile phase. Fig. 2 shows that the selected chromatographic conditions allow the complete separation of octopamine (t_R = 3.65 min) from tyramine (t_R = 4.85 min), and this separation was obtained even in the presence of a 1000-fold concentration of tyramine. The peak height was lowered

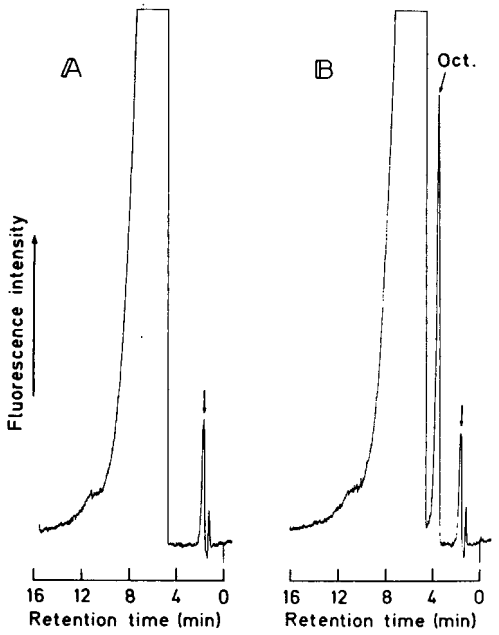

Fig. 2. HPLC fluorescence elution pattern of 100 μl supernatant from an incubation mixture in the assay of dopamine β-monooxygenase activity of a human serum. (A) Zero-time control, and (B) separation of octopamine (Oct., t_R = 3.65 min) from tyramine (t_R = 4.85 min). The arrows indicate the solvent front. 100 μl of the diluted (16 times) incubation mixture were injected into the liquid chromatograph; λ_{ex} = 280 nm and λ_{em} = 303 nm. For experimental details, see text.

by approx. 10% only when the same amount of octopamine was injected in 100 μl compared with 20 μl, with no loss in resolution.

Linearity and sensitivity

A linear relationship was obtained between the injected amount of octopamine and the peak height (r = 0.99) or the integrated peak area (data not shown). The limit of detection was approx. 50 pmoles octopamine (signal-to-noise ratio of 5).

Assay of dopamine β-monooxygenase in human serum

The useful application of the HPLC method to the assay of dopamine β-monooxygenase activity is most clearly shown by the assay of the enzyme in human serum. The hydroxylation of tyramine to octopamine by dopamine β-monooxygenase in human serum of high activity is shown in Fig. 2. None of the endogenous substances of the serum or components of the incubation mixture interfere with the chromatographic separation and the fluorimetric detection of octopamine, which is completely separated from the substrate to be hydroxylated (tyramine). Furthermore, no interference from compounds with high retention times was observed in multiple analyses in sequence.

In the standard assay procedure (pH 6.0) the amount of octopamine formed was proportional to the reaction time up to approx. 30 min (Fig. 3A) and to the amount of enzyme (serum) added (Fig. 3B). This result is in contrast

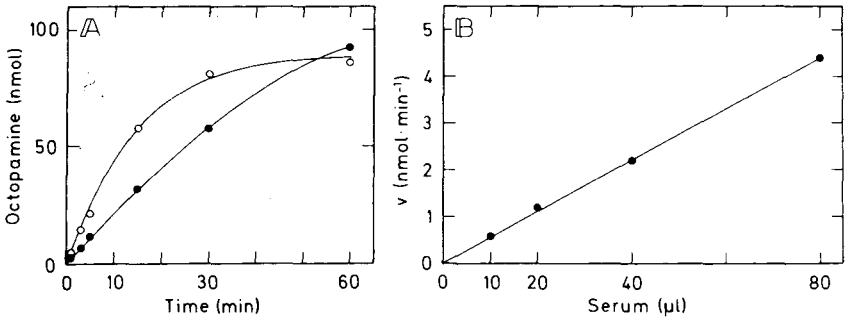

Fig. 3. (A) Time course of tyramine hydroxylation catalysed by dopamine β-monooxygenase of a human serum at pH 6.0 (•) and pH 5.0 (○). The ordinate indicates octopamine formed by 40 µl serum in 400 µl incubation mixture. (B) Effect of increasing amounts of enzyme (serum) on the rate of tyramine hydroxylation. The amount of octopamine formed was measured at pH 6.0 with a fixed incubation time (t = 30 min). The rate (v) was expressed as nmole • min^{-1}. For experimental details, see text. Incubation volumes were 400 µl.

to that obtained at pH 5.0 where the time course (Fig. 3A) is non-linear. It should also be mentioned that under our standard incubation conditions (pH 6.0, serum added) standard octopamine was found to be perfectly stable during an incubation period of 1 h, and the recovery was 100%. The reproducibility of the method was found to be better than ± 1% (S.D.).

A close correlation was found between the enzymatic activity of purified dopamine β-monooxygenase when the HPLC method was compared with the periodate oxidation method [10] (data not shown). When applied to sera from healthy adult subjects, the present HPLC method gave enzymatic activities which were in a range comparable to that obtained by a previously published method [5].

DISCUSSION

A recent trend in the microanalysis of biogenic amines has been the development of advanced HPLC methods for the rapid, specific and sensitive assay of the different amines and their metabolites from the same sample of biological material [1—4]. The improvements in sensitivity, specificity and versatility over previously used methods [for review, see ref. 8] are well-documented [1—4], and at the present stage of development the critical problem of this chromatographic procedure is the sensitivity of detection of the amines and the application as a routine method.

The recent developments of electrochemical detectors [1] as well as of continuous fluorescence monitoring, using *o*-phthalaldehyde as the fluorescence labelling reagent [5], have increased the sensitivity compared to UV detection [2—4]. Under favourable conditions these two methods allow the measurement of picomole amounts of amines eluted from HPLC columns. In the present study, however, detection based on the native fluorescence of octopamine has been developed to give an equivalent sensitivity, i.e. the detection limit is better than 50 pmoles.

The use of native fluorescence has obvious advantages over the two other detection methods. First, it is more specific since the different groups of

biogenic amines have their characteristic fluorescence spectra. Thus, detection by native fluorescence can be applied to a wide range of catecholamines and their biosynthetic precursors. Secondly, the method can be adapted to all commercially available spectrofluorometers now supplied with micro flow-cells. Finally, when used in combination with fully automated equipment (auto-injector), this method allows routine determination of 50 pmoles of octopamine with an efficiency of 6 analyses per h.

The useful application of the technique to the assay of dopamine β-monooxygenase activity in human serum is also demonstrated in the present study, including the assay conditions necessary to ensure linearity as a function of time and serum concentration. The method allows convenient and accurate measurements of low activities of dopamine β-monooxygenase in human serum, and presents advantages compared with other assay methods. The samples after incubation require little preparation, i.e. only precipitation of protein; the time required for a complete chromatogram is less than for the system based on reversed-phase partition chromatography [5], and no derivatization is required before detection.

The method presented in this report, although somewhat less sensitive than current radiochemical assay methods, offers advantages due to its speed, low cost, and avoidance of coupled enzyme reactions (see ref. 11 for references to methods and a discussion of problems in interpretation of dopamine β-monooxygenase assays). The sensitivity of our method, however, can be increased with the use of labelled tyramine. The advantage of the present chromatographic method is the more rapid separation of substrate and product compared with previously published HPLC methods [1—4].

ACKNOWLEDGEMENTS

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CHROMBIO. 209

RAPID AND SENSITIVE DETERMINATION OF ENZYMATIC DEGRADATION PRODUCTS OF ISOMERIC CHONDROITIN SULFATES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The separation and quantitative analysis of enzymatic degradation products of isomeric chondroitin sulfates by high-performance liquid chromatography (HPLC) are described. The substituted unsaturated disaccharides which result from digestion of chondroitin sulfates with chondroitinase are quickly separated on polar adsorbents such as silica gel. The UV absorption properties of these unsaturated disaccharides permit UV measurement with detection limits of approximately 100 ng. Their separation by HPLC facilitates the use of enzymatic methods for the determination of chondroitin sulfates A, B and C.

The potential of this method in clinical application is demonstrated by quantitative assays of glycosaminoglycans from a normal urine and urine from a patient with Hunter syndrome. The results are consistent with amount of isomeric chondroitin sulfates found in comparable urines by others.

INTRODUCTION

Chondroitin sulfates A, B and C** are isomeric mucopolysaccharides containing alternating hexuronic acid and N-acetylgalactosamine residues as the

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**The abbreviations used are: ChS-A, ChS-B, and ChS-C = chondroitin sulfates A (chondroitin 4-sulfate), B (dermatan sulfate), and C (chondroitin 6-sulfate); GAG = glycosaminoglycan(s) [mucopolysaccharide(s)]; CPC = cetylpyridinium chloride; ΔDi-4S = 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulfo-D-galactose; ΔDi-6S = 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-6-O-sulfo-D-galactose; ΔDi-OS = 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosylurohic acid)-D-galactose; ΔDi-diS_B = Δ4.5-sulfoglucuronido-acetylgalactosamine-4-sulfate.

characteristic structural backbone. The sulfate residue is located at C-4 of the galactosamine residue in ChS-A and B and at C-6 in ChS-C [1, 2]. The hexuronic acid residue is derived from glucuronic acid in ChS-A and C and from both iduronic acid and glucuronic acid in ChS-B [3—5]. Because of the similarity in structure of the three isomeric chondroitin sulfates, few convenient methods have been reported for the quantitative analysis of the individual isomers in mixtures [6—9]. An enzymatic method for the determination of these chondroitin sulfates was developed by Saito et al. [10], who purified chondroitinase ABC from *Proteus vulgaris* and chondroitinase AC from *Flavobacterium heparinum* which both specifically degraded chondroitin sulfates into the unsaturated disaccharides [11].

The same 4,5-unsaturated disaccharide 4-sulfate (ΔDi-4S) is formed from both ChS-A and ChS-B. The 4,5-unsaturated disaccharide 6-sulfate (ΔDi-6S) is produced only from ChS-C [10]. The assay of the unsaturated disaccharides in the enzymatic degradation product mixture by analytical procedures leads to the identification and measurement of glycosaminoglycans. This enzymatic method has been widely used to estimate glycosaminoglycans in tissues and in serum, urine and other body fluids [12—16].

Methods for the determination of the unsaturated disaccharides from the enzymatic degradation mixture have usually employed paper chromatography [10, 17, 18], and have required up to 36 h for the development of the chromatograms. The detection limit is approximately 25 μg. Recently, thin-layer chromatography has been employed for these assays [19]. Although the analysis was improved by shortening the chromatograph developing time and increasing detection sensitivity, the method required 12 h including a desalting step. These procedures did not provide detailed quantitative data. Colorimetric methods for the assays of sulfated disaccharides in the enzymatic digest by forming chromogens with both sulfated disaccharides and with only ΔDi-6S were also reported recently [20]. This method appears to be sensitive but does not effectively differentiate between the individual disaccharides. The HPLC method described here is rapid and sensitive and can be used to estimate quantities of each of the different chondroitin sulfates.

EXPERIMENTAL

Materials

Chondroitin sulfates A and B and the unsaturated disaccharides were purchased from Miles Labs. (Elkhart, Ind., U.S.A.). Chondroitin sulfate C was purchased from Calbiochem (San Diego, Calif., U.S.A.). Chondroitinase ABC and AC were obtained from both Miles Labs. and Sigma (St. Louis, Mo., U.S.A.).

Prepacked HPLC columns, Partisil PXS, 10 μm, 25 cm × 4.6 mm I.D. (Whatman Labs., Clifton, N.J., U.S.A.) and SI-5A, 5 μm, 25 cm × 4.6 mm I.D. (Brownlee Labs., Berkeley, Calif., U.S.A.) were employed. All solvents used were distilled in glass (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.).

Instruments

For HPLC determination, a Waters Assoc. Model 6000A liquid chromato-

graph equipped with a Waters Assoc. Model 440 UV monitor (254 nm) and a Waters Assoc. Model U6K injector were used. Peak heights and peak areas were determined with a Columbia Model Super 1 integrator (Columbia Sci. Ind., Austin, Texas, U.S.A.).

Isolation of urinary glycosaminoglycans

The urine specimens were filtered through Whatman No. 2 filter paper and adjusted to pH 5.0 with 0.5 *M* acetic acid. Two milliliters of the treated urine was mixed with 30 μl of 5% cetylpyridinium chloride (CPC) in 0.9% NaCl. The urinary GAG required 12 h equilibration at 4° with CPC for maximum precipitation as a CPC-polysaccharide complex [21]. After standing overnight at 4° the precipitate which formed was washed three times with 0.1% CPC and dissolved in 1 ml 2.0 *M* NaCl. The insoluble material was centrifuged. Four volumes of absolute ethanol were added to the supernatant and GAG were precipitated at 0° for 4 h. The precipitate was washed successively with 80% ethanol, absolute ethanol and ether, and dried under a stream of nitrogen. The GAG obtained were further dissolved in 0.5 ml distilled water and precipitated overnight at 0° in four volumes of absolute ethanol saturated with sodium acetate. The precipitate was washed with absolute ethanol and ether and dried in vacuum over P_2O_5. The GAG isolated was then used for enzymatic digestion.

Enzymatic digestion

The digestion mixture contained 10 μl of a solution of 100 μg standard GAG or GAG from 2 ml of urine in water, 10 μl of enriched Tris buffer (pH 8.0) [10] and 20 μl of an aqueous solution of either chondroitinase ABC or chondroitinase AC (10 units/ml). After incubation at 37° for 2.5 h, another 10-μl portion of enzyme solution was added and incubation continued for another 2.5 h at 37° [19]. The enzymes were omitted in the blank. Four volumes of absolute ethanol were then added and the mixtures left overnight at 4°. The clear supernatant obtained by centrifugation was dried under a stream of nitrogen and the residue, dissolved in 100 μl of 90% methanol, was applied on the HPLC instrument.

HPLC Separation of the disaccharides in the enzymatic digest

Columns packed with Partisil 10 (10 μm silica gel particles) and LiChrosorb SI-100 (5-μm silica gel particles) were used. The mobile phase consisted of a three-component mixture: dichloromethane—methanol—ammonium formate buffer. The enzymatic degradation products in 90% methanol were injected directly onto the column.

The ultraviolet absorption spectra of the products of chondroitinase action on chondroitin sulfates as a function of pH were studied by Nakada [22]. At a pH 1.8, an absorption maximum at 232 nm was recorded. Because of relatively strong and broad absorption, the unsaturated disaccharides can be measured with good sensitivity at 254 nm.

Separations were carried out isocratically at room temperatures. Details are given separately with each chromatogram.

RESULTS AND DISCUSSION

Chromatography

A variety of solvent systems were tested. The best separations were achieved with the ternary solvent dichloromethane—methanol—0.5 *M* ammonium formate buffer pH 4.8 (60:34:6, v/v/v). A separation of the three standard unsaturated disaccharides ΔDi-0S, ΔDi-6S and ΔDi-4S requires 20 min and is shown in Fig. 1.

The capacity ratio (k') values of the three disaccharides with different content of buffer are shown in Table I. The data demonstrate that a change of 1% in buffer content has a significant effect on peak resolution. It is apparent that the resolution of ΔDi-0S and ΔDi-6S increases and resolution of ΔDi-6S and ΔDi-4S decreases with increasing buffer content.

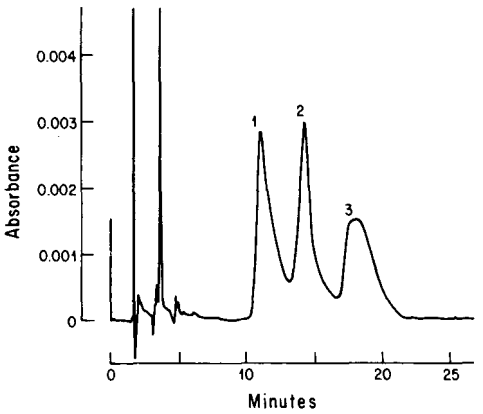

Fig. 1. HPLC of the three standard unsaturated disaccharides. 1 = ΔDi-OS; 2 = ΔDi-6S; 3 = ΔDi-4S; Solvent system: dichloromethane—methanol—0.5 *M* ammonium formate (pH 4.8) (60:34:6, v/v/v); Column: LiChrosorb SI-100, 5 μm, 25 cm × 4.6 mm I.D. Flow-rate: 2.0 ml/min. Pressure: 1800 p.s.i.; Injection port amount: 5 μg of each disaccharide. UV detection at 254 nm, 0.01 a.u.f.s.

TABLE I

CAPACITY FACTORS MEASURED FOR THE THREE STANDARD UNSATURATED DISACCHARIDES AT DIFFERENT AMOUNTS OF AQUEOUS BUFFER IN THE MOBILE PHASE

Values given are mean k' values determined from five separate chromatograms. Column: LiChrosorb SI-100, 5 μm, 25 cm × 4.6 mm I.D. Flow-rate: 2.0 ml/min. Pressure: 1800 p.s.i. UV detection at 254 nm, 0.01 a.u.f.s.

Disaccharide	Ratio of dichloromethane—methanol—0.5 *M* ammonium formate (pH 4.8)		
	60:34:4	60:34:6	60:34:7
ΔDi-0S	5	5.17	4.61
ΔDi-6S	6.11	6.89	6.39
ΔDi-4S	9.11	9.17	7.78

The pH of the ammonium formate buffer also plays an important role in the separation. When the pH was below 3.8, two peaks were observed for each disaccharide which suggests that tautomeric forms were separated. The phenomenon is reversible. At a higher pH of the buffer, only one peak for each disaccharide was observed. Peak shapes become more symmetric as the pH is increased. Above pH 4.0, the characteristic resolution of the three disaccharide peaks is nearly constant.

The dependence of the k' values on methanol content for the three unsaturated disaccharides is shown in Fig. 2. Ternary solvent systems containing less than 30% methanol separated into two phases. With an increase in the methanol content, ΔDi-6S was shifted toward ΔDi-0S, which was eluted first and eventually overlapped with it. The k' values decrease with increasing methanol concentration and asymptotically approach a limit.

Quantitation

For quantitative analysis, calibration curves were established at 254 nm for ΔDi-0S, ΔDi-4S and ΔDi-6S. The linearity is excellent over a large concentration range and the plots go through the point of origin. The advantage

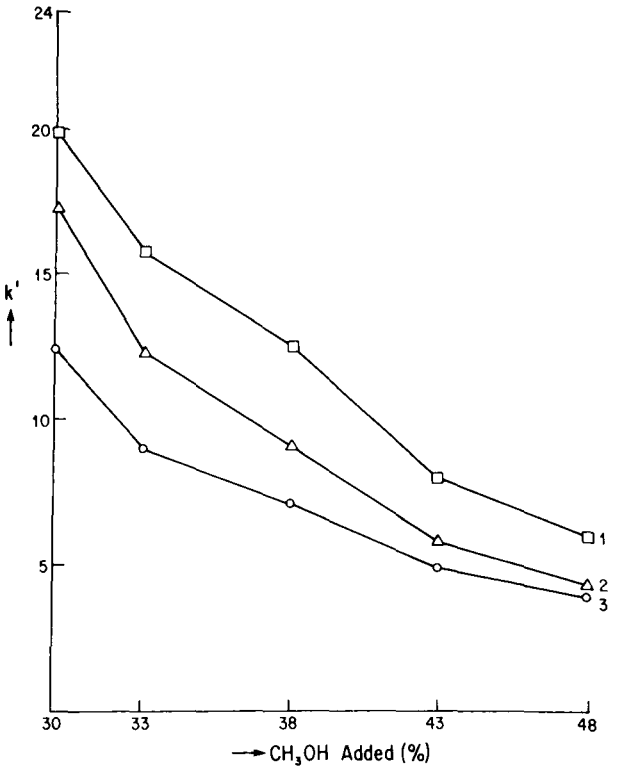

Fig. 2. k' Values of the three standard disaccharides as a function of the methanol content of the mobile phase. 1 = ΔDi-4S; 2 = ΔDi-6S; 3 = ΔDi-0S; Solvent system: dichloromethane—1 *M* ammonium formate (pH 4.8) (55:7) with different amounts of methanol added. Column: Whatman Partisil PXS, 10 μm, 25 cm × 4.6 mm I.D. Flow-rate: 2.0 ml/min. Pressure: 800 p.s.i. Injection volume: 15 μl. UV detection at 254 nm, 0.01 a.u.f.s.

of HPLC for quantitative separation is that it is possible to determine products directly from a calibration curve. In paper or thin-layer chromatography, several steps are required after developing the chromatogram to recover and assay the disaccharides [10, 19].

Application to enzyme digest

The potential of the HPLC separations of enzymatic degradation products is demonstrated in Fig. 3. Figs. 3A and 3C illustrate separations of the degradation products obtained in the incubation of ChS-A and ChS-C respectively with chondroitinase ABC. Figs. 3D and 3F illustrate separations after digestion of these with chondroitinase AC. The major peaks correspond to the expected products. Because of structural similarities, it is difficult to achieve a homogeneous preparation of one chondroitin sulfate to give a single disaccharide upon exhaustive digestion with chondroitinase. Thus, degradation of ChS-A

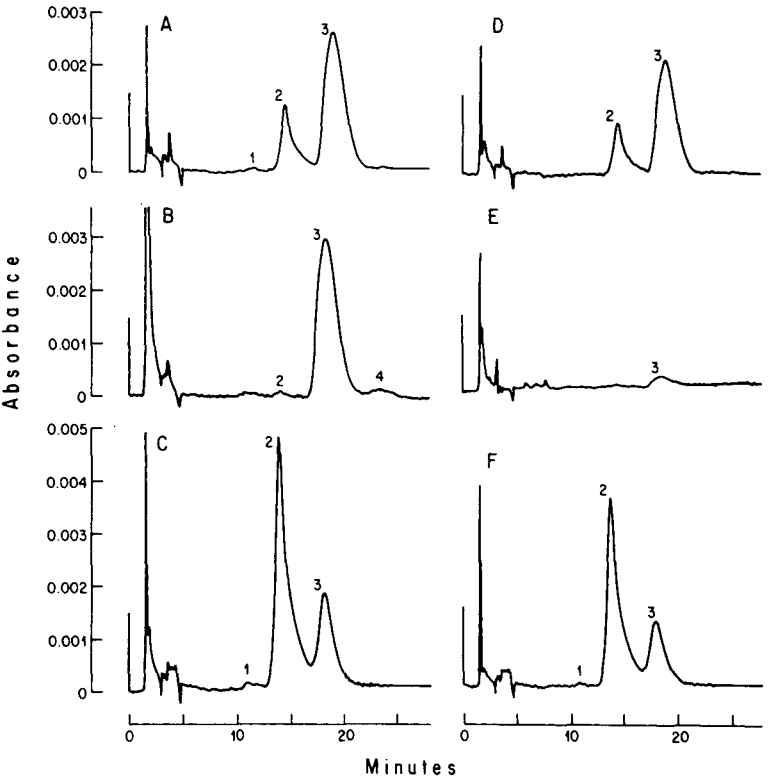

Fig. 3. HPLC of degradation products of chondroitin sulfates with chondroitinase. (A) 15 μg ChS-A with chondroitinase ABC; (B) 15 μg ChS-B with chondroitinase ABC; (C) 15 μg ChS-C with chondroitinase ABC; (D) 10 μg ChS-A with chondroitinase AC; (E) 10 μg ChS-B with chondroitinase AC; (F) 10 μg ChS-C with chondroitinase AC. Peaks: 1 = ΔDi-0S; 2 = ΔDi-6S; 3 = ΔDi-4S; 4 = ΔDi-diS_B. Solvent system: dichloromethane—methanol—0.5 *M* ammonium formate (pH 4.8) (60:34:6). Column: LiChrosorb SI-100, 5 μm, 25 cm × 4.6 mm I.D. Flow-rate: 2.0 ml/min. Pressure: 1800 p.s.i. UV detection at 254 nm, 0.01 a.u.f.s.

with chondroitinase ABC or AC gave some ΔDi-6S represented by additional minor peaks, which indicated possible contamination with ChS-C. Likewise, contamination with ChS-A in ChS-C preparations was suggested by the chromatogram.

Fig. 3B shows that ChS-B gave, as expected, ΔDi-4S as the major product following incubation with chondroitinase ABC. The last peak in this chromatogram (peak 4) is probably a disulfated disaccharide (ΔDi-diS_B) reported to occur in ChS-B [12, 23]. It has a higher k' than the monosulfated disaccharides as expected because of the polarity of the sulfate group. Positive identification of this peak is in progress. A chromatogram of the products of digestion of ChS-B with chondroitinase AC is shown in Fig. 3E. A small ΔDi-4S subunit is detected. Since chondroitinase AC is reported to have no activity toward ChS-B [11], this ΔDi-4S subunit could be due to contamination by ChS-A. A small peak corresponding to ΔDi-6S is observed when a large amount of enzyme digest (30% of 100 μg substrate) is injected which indicates contamination by a small amount of ChS-C is also possible. Blank runs indicate that disaccharides are not produced by this procedure in the absence of enzymes.

The recoveries of disaccharides from the enzymatic digest of 100 μg of chondroitin sulfates measured by HPLC are given in Table II. At least 70% of ChS-A can be recovered as disaccharides by digestion with chondroitinase ABC and up to 80% of ChS-A is recovered by action of chondroitinase AC-II, an enzyme of chondroitinase AC type.

TABLE II

RECOVERY OF CHONDROITIN SULFATES AFTER INCUBATION WITH CHONDROITINASE BY USING HPLC SEPARATION

Chondroitin sulfates (100 μg) A, B and C were incubated separately with chondroitinase ABC and AC as described in Experimental. The resulting products were dissolved in 90% methanol and injected onto the HPLC column. Values obtained are the mean of four sets from each digestion product.

Enzyme	Substrate	Product detected (μg)				Recovery (%, w/w)
		ΔDi-0S	ΔDi-6S	ΔDi-4S	ΔDi-diS_B	
Chondroitinase ABC	ChS-A	1.1	17.1	52.9	—	71.1
	ChS-B	—	1.1	59.2	N.M.*	> 60.3
	ChS-C	1.7	63.2	35.5	—	100.4
Chondroitinase AC-II**	ChS-A	1.4	19.5	63.1	—	84
	ChS-B	—	0.5	3.9	—	4.4
	ChS-C	1.7	70.8	36.2	—	108.7

*N.M. = Not measured.
**An enzyme of chondroitinase AC type.

Identification of urinary GAG

The application of HPLC to clinical assay is demonstrated by identification of urinary GAG in normal individuals and in a patient with Hunter syndrome.

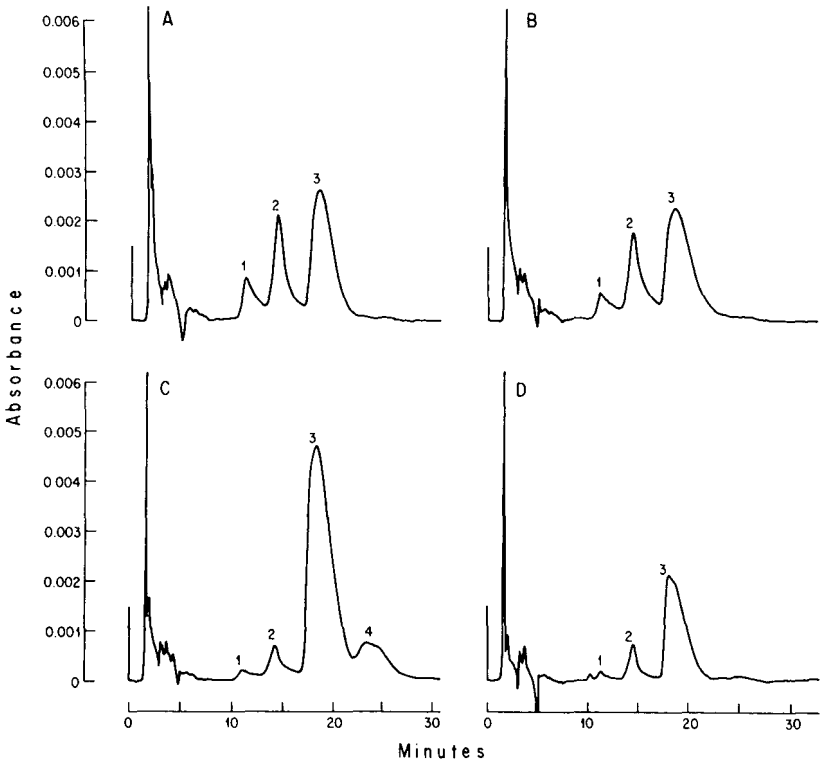

Fig. 4. HPLC of enzymatic degradation products of urinary GAG. (A) GAG from normal urine digestion with chondroitinase ABC; (B) GAG from normal urine digestion with chondroitinase AC; (C) GAG from Hunter's urine digestion with chondroitinase ABC; (D) GAG from Hunter's urine digestion with chondroitinase AC. Peaks: 1 = ΔDi-0S; 2 = ΔDi-6S; 3 = ΔDi-4S; 4 = ΔDi-diS. Solvent system: dichloromethane—methanol—0.5 *M* ammonium formate (pH 4.8) (60:34:6). Column: LiChrosorb SI-100, 5 μm, 25 cm × 4.6 mm I.D. Flow-rate: 2.0 ml/min. Pressure: 1800 p.s.i. UV detection at 254 nm, 0.01 a.u.f.s. Injection amounts are equivalent to 1.548 mg creatinine for normal urine and 0.234 mg creatinine for Hunter's urine.

Fig. 4 shows HPLC separations of disaccharides from urinary GAG after digestion with chondroitinase ABC and AC. The ΔDi-4S or ΔDi-6S obtained from normal urinary GAG are formed in the similar amounts in treatments with either enzyme chondroitinase ABC or AC (Figs. 4A and 4B). The results indicate that ChS-A and ChS-C predominate in the urinary GAG of normals and ChS-B is present in smaller amounts. This is consistent with the values obtained by other chromatographic methods. On the other hand, the patient with Hunter syndrome excreted a large amount of ChS-B [24]. The ΔDi-4S from Hunter's urinary GAG by digestion with chondroitinase AC are exclusively liberated from ChS-A (Fig. 4D), whereas ΔDi-4S obtained by digestion with chondroitinase ABC is liberated from ChS-A and B (Fig. 4C). The large difference in amounts of ΔDi-4S obtained from Hunter's urinary GAG in treatments with each to the two enzymes verifies that the excretion of large amounts of ChS-B is the characteristic of Hunter syndrome. Furthermore,

the appearance of the ΔDi-diS subunit in digestion of Hunter's urinary GAG with chondroitinase ABC (Fig. 4C) demonstrates the presence of ChS-B in the urine. Heparin sulfate is not detected by our chromatogram because of its resistance to digestion by the chondroitinase enzymes.

Table III shows the amounts of each disaccharide derived from enzymatic digestion of normal and Hunter's urinary GAG compared to excreted creatinine. The ratio of ΔDi-4S and ΔDi-6S from ChS-A and C are approximately 2:1 in normal urine. The urinary excretion of ChS-A and C from the patient with Hunter syndrome is significantly greater. These are essentially in agreement with previously reported enzymatic values [12].

TABLE III

ANALYSIS OF CHONDROITIN SULFATES IN URINE BY HPLC

Glycosaminoglycans were isolated from a normal child and a patient with Hunter syndrome and subjected to enzymatic digestion with chondroitinase ABC and AC as described in Experimental. The amount of disaccharides obtained in the enzymatic digest is given as μg disaccharides per mg of urinary creatinine.

Sources of urinary GAG	Enzyme used for degradation	Product	Amount of products (μg disaacharides per mg of urinary creatinine)	Type of chondroitin sulfate providing products
Normal urine	Chondroitinase ABC	1. ΔDi-0S	0.92	Non-sulfated chondroitin
		2. ΔDi-6S	2.72	ChS-C
		3. ΔDi-4S	4.98	ChS-A, ChS-B
	Chondroitinase AC	1. ΔDi-0S	0.62	Non-sulfated chondroitin
		2. ΔDi-6S	2.26	ChS-C
		3. ΔDi-4S	4.34	ChS-A
Hunter's urine	Chondroitinase ABC	1. ΔDi-0S	1.59	Non-sulfated chondroitin
		2. ΔDi-6S	6.11	ChS-C
		3. ΔDi-4S	60.15	ChS-A, ChS-B
		4. ΔDi-diS	N.M.*	ChS-B
	Chondroitinase AC	1. ΔDi-0S	1.51	Non-sulfated chondroitin
		2. ΔDi-6S	6.45	ChS-C
		3. ΔDi-4S	26.96	ChS-A

*N.M. = Not measured.

CONCLUSION

Effective separation and quantitation of the unsaturated disaccharides resulting from digestion of chondroitin sulfates with chondroitinase can be carried out by HPLC. This is a more rapid and sensitive determination than previously reported methods. The strong UV absorption of these disaccharides makes possible their detection at 254 nm without derivatization. The sensitivity can be increased by using a UV monitor at 232 nm.

Since it is possible to identify and distinguish between many of the individual mucopolysaccharidoses by the pattern of urinary GAG excretion we believe the present procedure could be usefully employed in such diag-

noses. Further work on the application of this technique to the quantitative determination of isomeric chondroitin sulfates is in progress.

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Biomedical Applications

CHROMBIO. 198

SIMPLIFIED METHOD FOR MONITORING TRICYCLIC ANTIDEPRESSANT THERAPY USING GAS—LIQUID CHROMATOGRAPHY WITH NITROGEN DETECTION

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SUMMARY

A simplified gas chromatographic method for the rapid measurement of tricyclic antidepressant drugs in plasma using a nitrogen-sensitive detector is described. All drugs are extracted and chromatographed under identical conditions. Tertiary amines are separated from their secondary amine metabolites, which are determined simultaneously without the need for derivatisation. The lower limit of accurate determination for most drugs is 10 μg/l.

The method has been applied to the routine measurement of amitriptyline and nortriptyline in plasma from patients receiving antidepressant treatment. Large and important interindividual differences in plasma concentrations in the patients investigated have been found, and the significance of these results is discussed.

INTRODUCTION

Many studies have shown that patients receiving similar doses of tricyclic antidepressant drugs exhibit a wide (10—20-fold) interindividual variation in steady-state plasma concentrations achieved [1—6]. Relationships between these concentrations and therapeutic response have been proposed [4, 5, 7—13] and there is also an increased incidence of toxic side-effects associated with high plasma concentrations [14—16]. It is important therefore, that rapid and sensitive methods should be available for the routine measurement of therapeutic concentrations of the commonly used tricyclic antidepressants.

Over recent years, a large number of gas chromatographic methods have been reported, most of which have concentrated on the most popular antidepressant, amitriptyline, and its demethylated metabolite, nortriptyline. The earliest methods used flame-ionization detection [17—19], but were relatively insensitive, requiring large volumes (5—10 ml) of plasma.

Electron-capture detection has been found to greatly increase sensitivity, and has provided reliable methods for nortriptyline [20—22]. However, polyfluorinated derivatives must be prepared, which is extremely time consuming.

Further, since tertiary amine antidepressants do not derivatise without prior demethylation, the technique has only a limited routine application.

A significant advance in methodology has been brought about by the introduction of "nitrogen-specific" detectors [23—31]. Some of these methods still involve a derivatisation procedure [23, 24, 26, 28] making them unsatisfactory for routine use. Several of these methods have specified special pre-treatment of glassware [23—26] and large samples (>4 ml for duplicate analyses) are still required by some assays [25, 26, 31] making them suitable only for research purposes.

Sensitivity and selectivity have been dramatically improved by the use of mass fragmentography [32, 33], but this expensive and highly specialised equipment is not available in the average laboratory. Immunological assays are still in the early stages of development, and problems of antibody cross-reactivity with other drugs and metabolites remain to be solved [34].

We describe here an improved method using gas—liquid chromatography (GLC) with nitrogen detection which has been in regular routine use for over a year for the measurement of plasma antidepressant concentrations in patients receiving medication with amitriptyline, nortriptyline, imipramine and clomipramine.

EXPERIMENTAL

Reagents

Reagents used were: absolute ethanol (GPR grade); borate buffer, pH 10 (Fisons, Loughborough, Great Britain); *n*-butyl acetate (Fisons; AnalaR grade); *n*-hexane (Fisons; Distol grade); 4 *M* NaOH (AnalaR) prepared freshly each week and 0.1 *M* H_2SO_4.

Glassware

Extraction tubes are glass with ground glass stoppers and are rinsed with ethanol just prior to use to reduce drug adsorption onto glass surfaces. The micro tubes (10 cm × 6 mm I.D.) are glass freeze-dry ampoules (FBG-Trident, Bristol, Great Britain). These are discarded after use.

Internal standard

Maprotiline hydrochloride, 2 μg/ml free base equivalent in aqueous solution was prepared from a concentrated acidic stock solution stored at 4°. For the determination of maprotiline an aqueous solution of nortriptyline hydrochloride (1 μg/ml) was used as an internal standard.

Standards

Plasma standards were prepared by spiking fresh bovine plasma to concentrations within the range 50—500 μg/l. These were stored deep frozen in 2-ml aliquots until required.

Extraction procedure

The plasma samples (1 ml) were added to buffer (1 ml) containing internal standard (150 μl) in a 10-ml stoppered glass tube. The drugs were extracted

into hexane (5 ml) by gentle mixing for 20 min. Extraction of drugs and internal standards was found to be maximal and constant after 15 min. Following centrifugation, the organic phase was transferred to a clean 10-ml glass tube containing sulphuric acid (1 ml) using a pasteur pipette rinsed in ethanol. After mixing for 10 min, the lower acid layer was transferred to a clean micro tube, then sodium hydroxide (100 μl) and butyl acetate (100 μl) were added. The tube was vortex mixed for 30 sec and centrifuged for 3 min. Aliquots (3—5 μl) of the upper organic phase were injected onto the chromatograph.

Chromatography

The gas chromatograph used was a Perkin-Elmer Model F33 equipped with an alkali flame nitrogen—phosphorus detector. The column (2 m × 2 mm I.D.) was of silanised glass packed with 3% SP 2250 on Supelcoport (80—100 mesh; Supelco, Bellefonte, Pa., U.S.A.). The column temperature was 250° and the carrier gas was argon at an inlet pressure of $3 \cdot 10^5$ N/m^2. The injection port temperature was 300°, with hydrogen pressure at $8 \cdot 10^4$ N/m^2 and an air pressure of $6 \cdot 10^4$ N/m^2. The detector bead setting ranged from 500 to 650 depending on the age of the bead.

Quantitation

A range of plasma standards were extracted simultaneously with the samples. The drug concentrations in the samples were calculated by comparison of peak height ratio to the internal standard. All standards and samples were run in duplicate, and repeated if the duplicates differed by more than ± 5%.

Blood samples

Blood samples (10 ml heparinised) were obtained from patients undergoing tricyclic antidepressant therapy. The plasma was separated and stored at 4° prior to analysis.

RESULTS

The absolute and relative retention times of a range of tricyclic antidepressants and related compounds are shown in Table I. As can be seen from this table, all the commonly prescribed antidepressant agents can be eluted on the system described. A number of tricyclic antidepressants (e.g., amitriptyline, imipramine, clomipramine and doxepin) produce active metabolites which can be resolved from their respective parent compounds.

Fig. 1 shows the resolution of imipramine and related compounds from the internal standard, maprotiline. Fig. 2 shows the resolution of amitriptyline and related compounds from maprotiline.

The sensitivity of the detector to the different compounds can be directly compared since each peak represents 10 ng on injection (Figs. 1 and 2).

Typical chromatograms obtained from the analysis of samples from patients receiving medication with amitriptyline and imipramine are shown in Fig. 3.

For the plasma standards, a good linear correlation was obtained between peak height ratio and concentration over the range 50—500 μg/1. Where plasma concentrations were expected to exceed this limit, smaller samples of plasma (0.2—0.5 ml) were used.

TABLE I

ABSOLUTE AND RETENTION TIME RELATIVE TO MAPROTILINE FOR TRICYCLIC ANTIDEPRESSANTS AND RELATED DRUGS

Drug	Retention time (min)	Retention relative to maprotiline
Butriptyline	3.2	0.56
Trimipramine	3.4	0.60
Amitriptyline	3.5	0.61
Imipramine	3.6	0.63
Doxepin	3.8	0.67
Zimelidine	3.9	0.68
Nortriptyline	4.0	0.70
Mianserin	4.1	0.72
Nomifensine	4.1	0.72
Desmethyltrimipramine	4.3	0.75
Protriptyline	4.4	0.77
Desmethyl imipramine	4.4	0.77
Desmethyl doxepin	4.6	0.81
Norzimelidine	4.8	0.84
Maprotiline	5.7	1.0
Clomipramine	6.5	1.14
Dothiepin	6.6	1.16
Desmethyl clomipramine	7.6	1.33
Northiaden	8.0	1.40
Dibenzepin	10.0	1.75

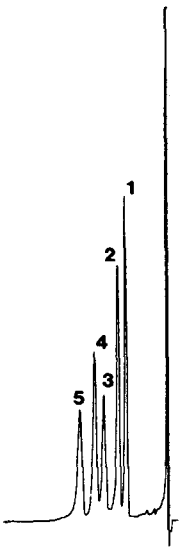

Fig. 1. The separation of imipramine (1), desipramine (2), maprotiline (3), clomipramine (4) and desmethyl clomipramine (5). Each peak represents 10 ng on injection.

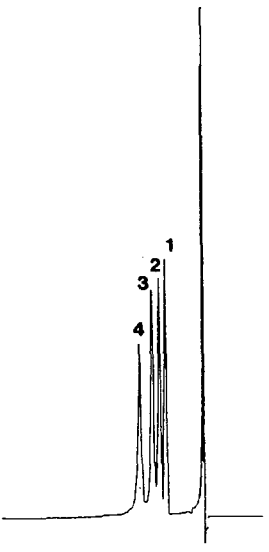

Fig. 2. The separation of amitriptyline (1), nortriptyline (2), protriptyline (3) and maprotiline (4). Each peak represents 10 ng on injection.

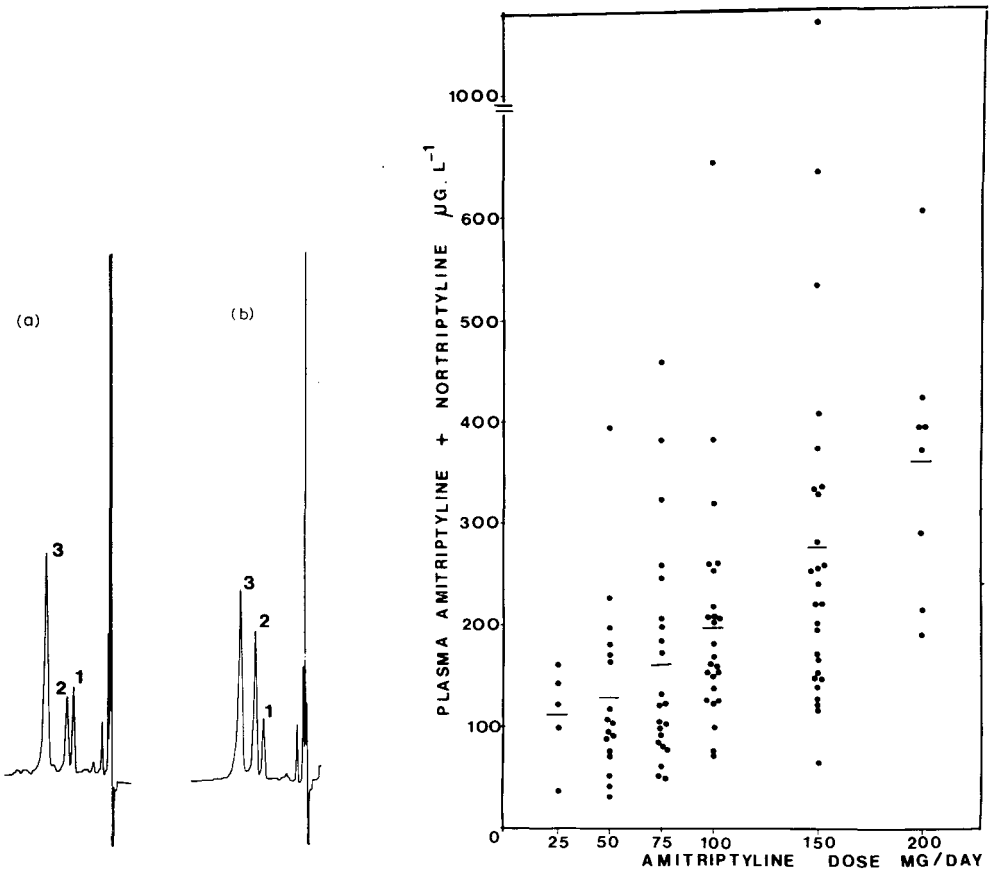

Fig. 3. (a) Trace obtained from the analysis of a plasma sample from a patient receiving amitriptyline, containing 45 μg/l amitriptyline (1) and 56 μg/l nortriptyline (2) with 3000 μg/l maprotiline internal standard (3). (b) Trace obtained from the analysis of a plasma sample from a patient receiving imipramine, containing 27 μg/l imipramine (1), and 112 μg/l desipramine (2) with 300 μg/l maprotiline internal standard (3).

Fig. 4. Relationship between daily dose and plasma concentration of amitriptyline plus nortriptyline in 103 patients receiving amitriptyline medication.

The reproducibility of the method was assessed by repeated analysis of pooled plasma samples taken from patients undergoing amitriptyiline medication. Table II shows the mean, standard deviation (S.D.), and coefficient of variation (C.V.) obtained from 20 replicate analyses of two different pooled samples. In all cases, a C.V. of less than 5% was achieved.

No interference with the assay by either endogenous plasma constituents or other commonly prescribed psychotropic drugs has yet been encountered.

Fig. 4 shows the distribution of amitriptyline plus nortriptyline plasma levels over a range of different doses obtained in the routine analysis of samples from 103 patients receiving amitriptyline treatment. It can be seen from Fig. 4 that mean plasma concentrations tended to increase with dosage.

TABLE II

MEAN, STANDARD DEVIATION (S.D.) AND COEFFICIENT OF VARIATION (C.V.) FOR 20 REPLICATE ANALYSES OF TWO POOLED SAMPLES OF PLASMA TAKEN FROM PATIENTS RECEIVING TREATMENT WITH AMITRIPTYLINE

Pooled plasma	Amitriptyline (μg/l)			Nortriptyline (μg/l)		
	Mean	S.D.	C.V.(%)	Mean	S.D.	C.V.(%)
"High" value	295	5.4	1.8	200	5.7	2.9
"Low" value	75	3.0	4.0	57	2.6	4.5

TABLE III

MEAN PLASMA AMITRIPTYLINE, NORTRIPTYLINE AND AMITRIPTYLINE–NORTRIPTYLINE RATIO IN 103 PATIENTS RECEIVING DIFFERENT DAILY DOSES OF AMITRIPTYLINE

Dose (mg/day)	*n*	Plasma conc. (μ/l), mean (S.D.)			
		Amitriptyline	Nortriptyline	Amitrityline plus nortriptyline	Amitriptyline/ nortriptyline, mean (S.D.)
25	5	48 (18)	65 (37)	113 (48)	0.88 (0.41)
50	17	74 (53)	57 (45)	130 (88)	1.65 (1.39)
75	22	84 (58)	81 (61)	162 (113)	1.15 (0.49)
100	24	57 (57)	95 (81)	197 (123)	1.53 (1.33)
150	27	132 (80)	145 (146)	276 (205)	1.25 (0.85)
200	8	173 (104)	173 (47)	359 (132)	1.00 (0.47)
All doses (mean) 103.6	103	105 (71)	103 (98)	209 (154)	1.32 (1.01)

However, large interindividual differences in plasma levels were seen in patients prescribed similar doses. Table III shows the mean and S.D. plasma amitriptyline plus nortriptyline concentrations for different daily doses.

The relationship between amitriptyline and nortriptyline concentrations in individual patients is shown in Fig. 5. Individual ratios of amitriptyline to nortriptyline plasma concentrations ranged between 0.3 and 6.6 (mean 1.3, S.D. 1.0). However, there was a highly significant correlation ($r = 0.63$, $p < 0.0001$) between amitriptyline and nortriptyline plasma concentrations as shown in Fig. 5.

DISCUSSION

Most of the commonly used antidepressant drugs and, where applicable, their demethylated metabolites, can be measured accurately in plasma at therapeutic concentrations by the procedure described above. Analytical time has been saved by eliminating the need for derivative formation prior to chromatography and by introducing a final micro-phase extraction step in

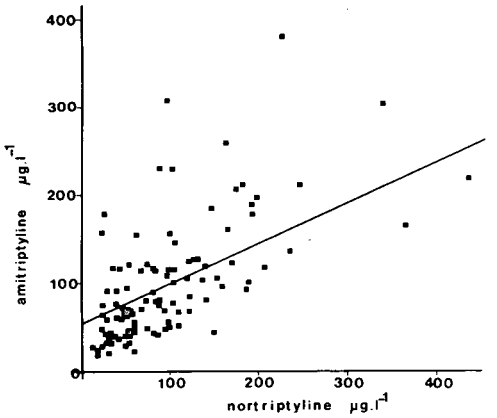

Fig. 5. Relationship between plasma amitriptyline (y) and nortriptyline (x) in 103 individual patients. The point representing a patient with a plasma AT and NT concentration of 311 and 758 µg/l, respectively, is not shown, but has been included in the regression analysis: $y = 0.46\,x + 56.2$ ($r = 0.63$, $p < 0.0001$).

place of the more usual procedure of extract concentration. Other than rinsing with ethanol, no special pre-treatment of glassware is required and the use of disposable tubes for the final extraction stage reduces the risk of contamination from recycled glassware.

The method has also been successfully applied to the measurement of antidepressant plasma concentrations in cases of overdosage. In such cases only 1 ml of plasma is required for duplicate analyses. This has been carried out for all the drugs shown in Table I, with the exception of zimelidine and its active metabolite norzimelidine. These measurements have been found to be useful in the diagnosis of suspected antidepressant poisoning in both adults and children.

The major application of the technique in this laboratory has been the routine analyses of drug plasma concentrations in patients receiving tricyclic antidepressant medication. This is demonstrated in the results obtained from 103 patients receiving treatment with amitriptyline which are shown in Fig. 4 and Table III. As can be seen in Fig. 4, extremely large interindividual differences in "steady-state" drug plasma concentrations are obtained in the patients receiving routine treatment with amitriptyline. Although mean plasma levels of amitriptyline and nortriptyline tended to increase with prescribed daily dose, much overlap is apparent. The plasma concentration ratio of amitriptyline to nortriptyline in individual patients ranged from 0.3 to 6.6 (mean 1.3, S.D. 1.0) overall, however, there was a highly significant correlation ($r = 0.63$, $p < 0.0001$) between amitriptyline and nortriptyline concentrations (Fig. 5). In view of the reported relationship between plasma concentrations of tricyclic antidepressants and their clinical effects [4, 5, 7—16] plasma concentrations achieved by patients during routine treatment may be extremely important. From the results shown in Fig. 4 for amitriptyline medication, it is reasonable to argue that a large proportion of patients may never achieve therapeutically effective drug levels, whereas others may have levels that

are likely to cause serious toxicity. Measurement of antidepressant plasma concentrations using rapid and simple assay procedures might be a more efficient way of tailoring drug dosage to suit individual patients' requirements.

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Biomedical Applications

CHROMBIO. 204

IMPROVED METHOD FOR THE RAPID DETERMINATION OF ISOSORBIDE DINITRATE IN HUMAN PLASMA AND ITS APPLICATION IN PHARMACOKINETIC STUDIES

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SUMMARY

A rapid, accurate and highly sensitive method was developed for the determination of isosorbide dinitrate in human plasma. Concentrations in the lower nanogram and subnanogram range are determined by a one-step extraction of 2 ml plasma, containing 4 ng/ml nitroglycerine as internal standard, with 5.5 ml *n*-pentane. The extract is subjected to gas—liquid chromatography—electron capture detection analysis. The lower limit of quantitation is 200 pg/ml, but concentrations as low as 50 pg/ml are still detectable. The method allows the quantitative determination of isosorbide dinitrate plasma levels in man following a 5 mg sublingual administration up to four hours after application.

INTRODUCTION

Polynitric esters which are used as vasodilators in the therapy of angina pectoris, are still pharmacologically effective in doses of only a few milligrams. In view of the fact that these substances are very quickly metabolized [1, 2] and the blood levels of the unaltered drugs are within the lower nanogram and subnanogram range, their quantitative analysis has to meet high requirements with regard to the sensitivity of detection and accuracy of the method.

For the detection of isosorbide dinitrate (ISD) in human plasma after administration of therapeutic doses, gas—liquid chromatography (GLC) has proved to be adequate [3—8], in addition to the use of ^{14}C-labelled isosorbide dinitrate [2]. While sensitivity of detection is inadequate with flame ionization detection [3, 4], the detection limits are scaled down to the picogram range with electron capture detection [5—8]. When gas chromatographic (GC) analytical procedures are applied in this concentration range, the necessary elimination of interfering plasma compounds by a preceding treatment of the sample often leads to a compromise between the sensitivity as

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well as accuracy and time-consuming experimental procedures.

The ISD—electron capture detection methods which have been described up to now, either are highly sensitive and accurate, but very time-consuming [6], or they are, if less time-consuming, less sensitive [8] or less reproducible [7]. Other methods available are not sufficiently characterized to demonstrate their application in the lower nanogram range [5, 8]. The present work describes an electron capture—GLC determination of ISD which combines particularly high sensitivity and good reproducibility as well as accuracy with a very simple and quick procedure of analysis. The total time consumption for processing and GC procedure amounts to 20 min. The method therefore is particularly well suited for the routine determination of large quantities of samples.

The validity of the method described is demonstrated by the determination of ISD plasma levels in human volunteers after sublingual administration of an ISD tablet that allowed the evaluation of some pharmacokinetic parameters.

EXPERIMENTAL

Reagents and materials

Spectroscopic grade *n*-pentane (Merck, Darmstadt, G.F.R.) was three times glass-distilled, the third distillation being shortly before use. Spectroscopic grade *n*-hexane (Merck) was three times glass-distilled; a fourth distillation was performed after refluxing for 2 h over liquid Na-K-alloy. Nitroglycerine (Merck) was used as a 1% solution in ethanol. ISD was available as a 50% preparation on powdered lactose base (Schweizer Sprengstoffabrik, Dottikon, Switzerland). The nitrate was extracted with *n*-pentane and purified by recrystallization. To avoid contamination of blood plasma by plasticizers from plastic materials during sampling and processing [9] the nitrate-containing probes were allowed to contact only glassware during the whole analysis.

Extraction procedure

In a stoppered 20-ml centrifuge tube, 2 ml of plasma were spiked with 8 ng of nitroglycerine as internal standard by adding 10 μl of a 0.8 μg/ml solution of nitroglycerine in *n*-hexane and agitating for a few seconds. To this 5.5 ml *n*-pentane were added and the tube was shaken for 40 sec. Immediately after shaking and without centrifugation the organic layer was transferred to a conical glass tube and carefully evaporated at room temperature under a gentle stream of nitrogen. The nitrogen was washed by passing it through liquid paraffin. To prevent evaporation of nitroglycerine the nitrogen stream was interrupted as soon as dryness was achieved. After addition of 100 μl of *n*-hexane to the residue, the solution was agitated for a few seconds and the samples were stored at 4° until injection into the gas chromatograph.

Electron capture GLC

A Packard Model 419 gas-chromatograph equipped with a 10 mCi ^{63}Ni electron capture detector (ECD) was used.

Argon—methane (95:5) was used as carrier gas with a flow-rate of 20 ml/min

through the column and as scavenger gas with a flow-rate of 35 ml/min through the detector. The gas was dried over a molecular sieve (Oxisorb F, WGA, Düsseldorf, G.F.R.). Installation of flow-controlling gas valves (DC 1400, Kontron, Munich, G.F.R.) instead of pressure-controlling valves resulted in a striking reduction of the baseline noise. Maximum sensitivity was obtained with the following temperature settings: oven 130°, injection port 165°, detector 180°. Under these conditions retention times were 50 sec and 165 sec for nitroglycerine and ISD, respectively. The detector was operated with an electron capture lineariser (Model 736, Packard) in the "constant current pulse mode" with a reference current of $1.75 \cdot 10^{-9}$ A.

The septum of the injector port was changed every 20 injections. The septum was pretreated by washings with *n*-hexane, followed by drying at 200° for 2 h. The injection volume was 4 µl and the injection was performed in duplicate. For injection, a 5 µl glass syringe (SGE, Melbourne, Australia) was used.

Quantitation

Quantitation of ISD was achieved by using nitroglycerine as internal standard. The peak heights of the two nitrates on the chromatogram were measured. From the ratio of the peak heights, obtained by analysing plasma samples to which were added known amounts of nitroglycerine and ISD, a calibration curve was constructed. To eliminate the influence of possible long time gradual variations of the calibration factor, the calibration was performed weekly.

Column

The glass columns, 1.2 m × 2.4 mm I.D., were silanized by treatment with a solution of 1% trimethylsilylchloride (Merck) in benzene for 1 h. After flushing with methanol, the column was air-dried and loaded with 3% silicone OV-101 on Gas-Chrom Q, 80—100 mesh (WGA). The column was primed before use by heating (100°) for 2 h under nitrogen flow, subsequent thermal aging at 340° without nitrogen flushing (no-flow-conditioning) overnight, and conditioning with carrier gas flow for 24 h at 270°. Priming without no-flow-conditioning could also be achieved, but in this case it takes over a week for the full sensitivity to be attained.

Plasma level study

Eight healthy volunteers (female, aged 18—50 years) received a 5-mg sublingual ISD tablet*. Before, as well as 6, 11, 16, 23, 31, 46, 61, 90, 120, 180 and 240 min after administration, ca. 10 ml blood were drawn from the cubital vein into heparinized glass syringes. In order to prevent further metabolism or disintegration of ISD in vitro, the blood was transferred from the withdrawal syringe into iced glass tubes (where 50 µl of 0.002 *N* silver nitrate solution was added) and immediately centrifuged at 1300 *g* in a refrigerated centrifuge at 4°. Addition of larger amounts of silver nitrate resulted in hemolysis. The plasma was either processed immediately for further analysis

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or deep frozen until the analysis was to take place (not later than 8 h after withdrawal of the sample).

RESULTS AND DISCUSSION

Sensitivity

With the described procedure chromatograms were obtained in which ISD and nitroglycerine appeared as symmetrical peaks which were hardly influenced by other plasma components or impurities. Fig. 1A shows a typical gas chromatogram of the nitrates extracted from human plasma. Fig. 1B shows a chromatogram of blank plasma from the same subject treated by the same procedure. The recording conditions for both chromatograms were: attenuation 1 X 64, 5 mV recorder operated at a charge speed of 600 mm/h.

Corresponding to a recording amplitude of 200 mm the signal heights of ISD and nitroglycerine are 150 mm and 170 mm respectively. The concentration of ISD was 5 ng/ml in plasma and that of nitroglycerine 4 ng/ml in plasma. This corresponds to amounts of 400 pg and 320 pg injected onto the column. The minimum detectable amount of ISD as a solution of the pure substance, was 2 pg (signal-to-noise ratio = 3:1).

Based on the extraction of 2 ml plasma the lower limit of detection of ISD in plasma was 50 pg/ml. In some cases ISD-free plasma showed "isosorbide dinitrate" peaks, which, however, never corresponded to more than 100 pg/ml. A plasma concentration of 200 pg/ml therefore was regarded as the lowest limit of quantitative determination.

A lowering of the GC response for nitroglycerine but not for ISD was observed, when more than approx. 45 min elapsed between two injections.

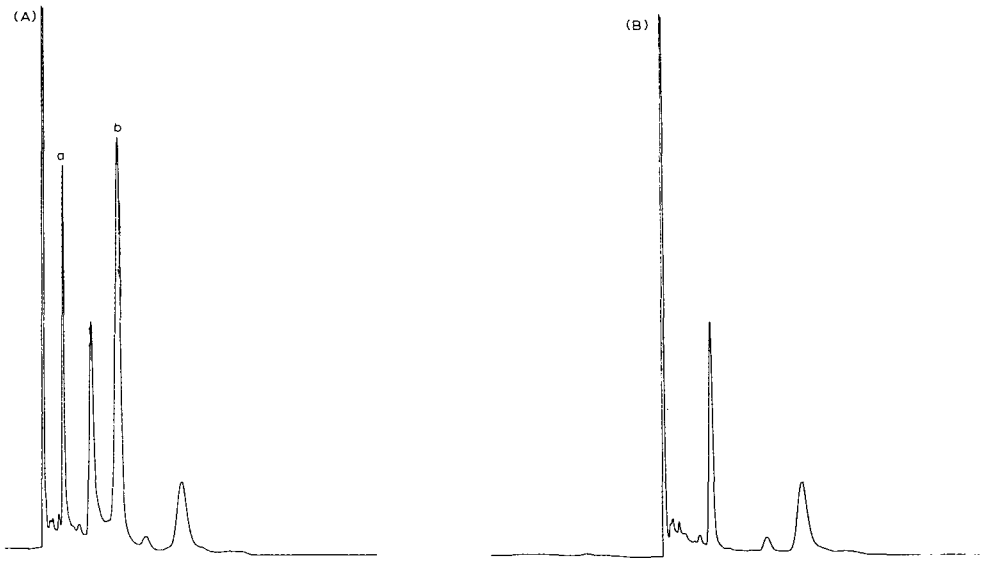

Fig. 1. Typical chromatograms obtained from human plasma extracts: A, plasma spiked with 4 ng/ml nitroglycerine and 5 ng/ml ISD. Peaks: a = nitroglycerine, b = ISD. B, blank plasma.

In this case full sensitivity could be re-established by two or three 1-μl injections of a 1-μg/ml solution of nitroglycerine. The lack of memory effects could be proven by injection of pure solvent following the injection of nitrates.

Exhaustion of the column was indicated by a loss of sensitivity for both nitrates and was observed after approx. 2000 injections.

Accuracy, reproducibility

Calibration curves were constructed by analysing samples to which different amounts of ISD were added to yield concentrations of 0.5, 1.0, 2.0, 5.0, 10.0 and 15.0 ng/ml of plasma. The concentration of the internal standard nitroglycerine was held constant (4 ng/ml). A linear relationship between the response ratios of the two nitrates and ISD concentrations was observed. Table I shows the measured response ratios of five calibration curves obtained during five different weeks. The coefficients of a linear regression analysis of the calibration values were found to be in the range from 0.9966 to 0.9998,

TABLE I

RATIOS OF THE PEAK HEIGHTS OF ISD AND NITROGLYCERINE

Obtained from plasma runs over several weeks.

ISD added (ng/ml)	Ratio of peak heights Weeks				
	1	2	3	4	5
0.5	0.036	0.042	0.024	0.052	0.049
1.0	0.082	0.069	0.066	0.097	0.095
2.0	0.159	0.165	0.143	0.179	0.195
5.0	0.451	0.456	0.426	0.444	0.541
10.0	0.996	0.892	0.962	0.915	1.034
15.0	1.428	1.398	1.336	1.365	1.550

TABLE II

REPRODUCIBILITY OF ISD DETERMINATION BASED ON SUCCESSIVE PLASMA RUNS

ISD plasma concentrations

Given (ng/ml)	Found Individual plasma runs (ng/ml)					Average (ng/ml)	Coefficient of variation (%)
1.0	0.88	1.05	0.83			0.92	13
1.0	0.89	0.95	0.98	1.04	0.91	0.95	6
2.0	2.28	2.18	2.33			2.26	3
4.0	4.83	4.50	4.80			4.71	4
5.0	5.01	5.81	5.45	5.25	5.35	5.37	5
10.0	9.96	11.36	10.27			10.53	7
15.0	16.43	14.98	15.44	15.64	15.02	15.50	4

thus indicating the high accuracy of the concentrations determined and the good linearity of the calibration curves. The reproducibility of the analysis as determined by repeated assays of plasma with known ISD concentrations is shown in Table II. Triplicate and quintuplicate assays were carried out in different months. The highest coefficient of variation found at a concentration of 1 ng/ml was 13%, corresponding to a standard deviation of ± 120 pg ISD per ml of plasma. Considering that assays reported previously involve coefficients of variation up to 50% in this lower concentration range, this result means a substantial improvement of the reliability of the measured values.

Recoveries

Total recoveries of nitroglycerine and ISD including all steps of the analytical procedure were determined by a comparison of the peak heights obtained from processed plasma samples with the peak heights of directly injected standards. Plasma samples with known ISD concentrations from 0.5 ng/ml to 10 ng/ml and nitroglycerine concentrations of 4 ng/ml as well as standard solutions of 100 ng/ml in *n*-pentane were used. The results (Table III) implicate that nitroglycerine is somewhat more readily extracted than ISD. However, there is no concentration-dependent trend in the recoveries observed. Variations between individual recovery values are mainly attributed to different volumes of *n*-pentane solution obtained by separating the organic layer from the aqueous phase. In addition the given recoveries also include accidental errors by variations in GC response.

Duration of assay

One of the goals of this work was to reduce as much as possible the time needed for ISD assays. Some characteristics of the described assay make it possible to dispense with time consuming purification procedures without obtaining interfering peaks in the chromatogram. The choice and high purity of the solvents *n*-pentane and *n*-hexane are the most essential characteristics of our method. The homopolar character of *n*-pentane and the short extrac-

TABLE III

TOTAL RECOVERY OF ISD AND NITROGLYCERINE ADDED TO HUMAN PLASMA

n = number of conducted analyses

	Amount added (ng/ml)	*n*	Recovery (%) (average ± S.D.)	Recovery (%) (total mean ± S.D.)
Isosorbide dinitrate	0.5	3	62 ± 9	
	1.0	3	71 ± 10	67 ± 10
	2.0	3	66 ± 4	
	4.0	3	65 ± 14	
	7.0	3	71 ± 7	
	10.0	3	71 ± 18	84 ± 18
Nitroglycerine	4.0	12	84 ± 18	

tion time of 40 sec prevent the formation of emulsions thus making it unnecessary to separate the phases by centrifugation. A further advantage of *n*-pentane is its rapid evaporation. As we use a small *n*-pentane volume of 5.5 ml for extraction, the total time for plasma processing can be reduced to 12 min. A comparison of chromatograms of processed plasma with and without added nitroglycerine, revealed that already a slight modification of the solvent properties can modify the result: the trace amount (0.8 μg) of ethanol derived from the undiluted nitroglycerine stock solution added by spiking the plasma with the internal standard, caused a remarkable increase of a peak with the retention time of 111 sec (largest peak in Fig. 1B) that could not be attributed to ethanol itself. The same increase was observed when we added 1 μg of ethanol to 2 ml of plasma prior to the extraction as performed to obtain the chromatogram of Fig. 1B.

Furthermore, the time needed for the assay would have been reduced by concentrating the *n*-pentane volume to 100 μl instead of evaporating it to dryness. But when such a solution was injected into the gas chromatograph we obtained a lot of additional peaks that partially interfered with the nitrate peaks. As the solvent properties of *n*-pentane and *n*-hexane are only slightly different, we suppose that the dried residue on evaporation is partially irreversibly adsorbed at the glass surface of the tube. Presumably this desirable effect will not occur when the glassware is silanized [6].

As 8 min are needed for the GC procedure, about 20 min are required for a complete ISD analysis. There are no delayed peaks in the chromatogram, as has been observed in previously reported assays [6]. Therefore, every 8 min another sample can be injected.

In vivo studies

Table IV shows the results of ISD plasma level determinations in human volunteers according to the method described. All plasma values were obtained by duplicate assays. The sensitivity of the method allowed detection of ISD

TABLE IV

PLASMA CONCENTRATIONS OF ISD (ng/ml) FOLLOWING A 5 mg SUBLINGUAL DOSE

N.D. = not detected

Time after dosing (min)	Subject								Mean ± S.D.
	1	2	3	4	5	6	7	8	
6	6.3	N.D.	0.8	7.6	13.7	1.0	1.9	2.5	4.2 ± 4.7
11	16.8	34.9	8.1	17.0	27.6	4.2	10.7	13.4	16.6 ± 10.2
16	15.5	42.1	5.2	13.1	—	5.0	10.5	29.0	17.3 ± 13.5
23	13.0	24.3	8.0	11.0	15.0	5.7	11.2	22.1	13.8 ± 6.5
31	10.1	18.6	6.7	8.5	13.5	5.2	9.7	16.9	11.2 ± 4.8
46	6.3	10.5	2.9	5.4	10.5	4.9	6.3	11.1	7.2 ± 3.1
61	4.2	8.0	3.0	8.6	5.7	3.7	9.5	5.8	6.1 ± 2.4
91	1.8	3.8	1.4	4.0	2.9	1.4	3.0	3.2	2.7 ± 1.0
120	0.9	1.9	0.3	1.2	1.4	0.7	0.9	2.8	1.3 ± 0.8
180	0.3	1.1	N.D.	N.D.	0.5	0.3	0.4	0.7	0.4 ± 0.4
240	0.2	0.3	N.D.	N.D.	0.3	0.3	0.2	0.2	0.2 ± 0.1

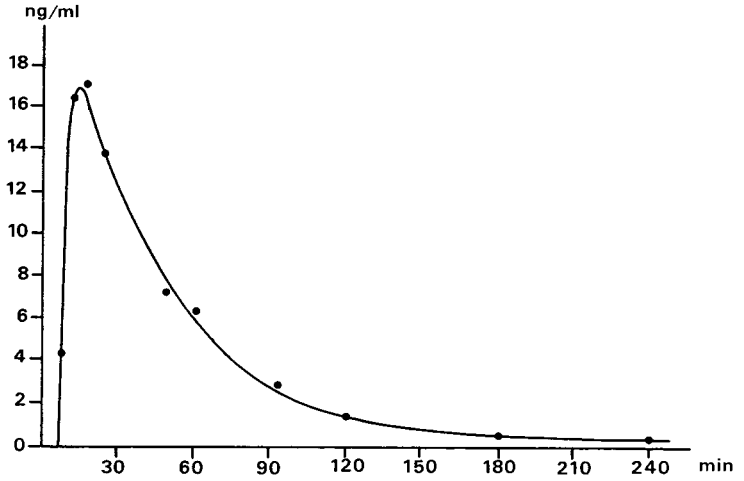

Fig. 2. Average plasma levels of ISD in 8 subjects after sublingual administration of a 5 mg tablet. The curve was calculated based on one compartment body model assumption.

in plasma even 3—4 h after ingestion of the tablet. The average plasma levels of all 8 volunteers are depicted in Fig. 2. ISD concentrations reached a maximum of 17.3 ng/ml within 16 min of administration. A pharmacokinetic one-compartment analysis of the average plasma level curve resulted in a mean half-life of 1.4 min for the absorption and 30.1 min for elimination of ISD. A lapse of approx. 5 min from ingestion of the tablet to the first detectable occurrence of ISD was calculated.

CONCLUSION

A rapid and accurate method for the determination of ISD in human plasma in the lower nanogram and subnanogram range was developed. The method, utilizing nitroglycerine as internal standard, is based on GLC analysis with a ^{63}Ni ECD. ECD assays of ISD have been reported previously. They either involved time-consuming multistep processing of the samples or a decreased accuracy in the lower nanogram range.

This assay combines a highly accurate and sensitive detection of ISD with a rapid experimental procedure. The sample processing is limited to essentially one step, carried out in 12 min. Total analysis including GC procedure can be performed in approx. 20 min.

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CHROMBIO. 205

DETERMINATION OF MOLSIDOMINE IN PLASMA BY HIGH-PERFORMANCE LIQUID COLUMN CHROMATOGRAPHY

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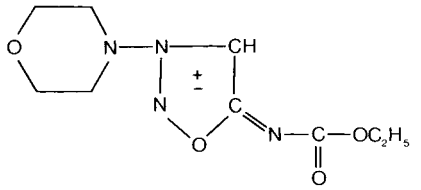
SUMMARY

A high-performance liquid chromatographic method is described for the analysis of the anti-anginal compound 5-ethoxycarbonyl-3-morpholinosydnonimine (Molsidomine) in human and dog plasma. The drug was extracted from plasma into chloroform and the analysis was carried out on a reversed-phase column, the column effluent being monitored by UV absorption at 312 nm. The method is sensitive (2 ± 0.3 ng/ml) and specific. The method was applied to a study in which human volunteers received an aqueous solution of the drug and then, on a separate occasion, a tablet formulation. Peak plasma levels of 20—30 ng/ml (tablet) and 10—19 ng/ml (aqueous solution) were obtained following a 2-mg oral dose.

INTRODUCTION

Molsidomine (5-ethoxycarbonyl-3-morpholinosydnonimine, developed by Takeda Chemical Industries, Osaka, Japan, and licensed to Casella Farbwerke Mainkur, Frankfurt, G.F.R.) is a novel sydnonimine derivative with a mesionic ring which has been shown to possess a sustained anti-anginal effect following oral treatment [1].



Molsidomine

There are no reports of chromatographic methods for the analysis of Sydnones and, in view of the polarity of the mesionic ring system, it was considered unlikely that a gas chromatographic method would be applicable. It was

decided, therefore, to develop a high-performance liquid chromatographic method which would be sufficiently sensitive and specific to follow the concentration—time course of Molsidomine in plasma following therapeutic doses (1—2 mg) of the drug to human volunteers.

This paper describes the development of such a method, using reversed-phase chromatography, and its application to a human volunteer study involving two different formulations of Molsidomine.

EXPERIMENTAL

Apparatus and materials

The chromatograph consisted of a pump (constant flow syringe pump; Applied Chromatography Systems, Luton, Great Britain), an injector (modified hoke valve stop flow injector; H.S.C.P., Bourne End, Great Britain) and a UV detector (CE 212 UV variable wavelength monitor; Cecil Instr., Cambridge, Great Britain) operated at 312 nm, the $\lambda_{max.}$ for Molsidomine (ϵ = 1.5×10^4). The separation column was a 125 × 4.5 mm I.D. stainless-steel tube packed with ODS Hypersil (5 μm diameter, 200 m^2/g; Shandon Southern, Runcorn, Great Britain). The mobile phase consisted of a mixture of equal volumes of 0.1 *M* sodium acetate aqueous solution and methanol.

All chemicals and solvents were of analytical-reagent grade (Fison's Scientific Apparatus, Loughborough, Great Britain) and were used without further pre-treatment. Stock solutions of Molsidomine were prepared in distilled water and stored in the dark at 0—4°.

Preparation of columns

The separation column was packed by a slurry technique as follows: the packing material (2 g) was added to a solution of 80% methanol in 0.1% (w/v) aqueous sodium acetate trihydrate (10 ml) and the mixture was ultrasonicated for 5 min. The column was packed by forcing the slurry into the column tube by means of a constant-pressure pump (MCP 71; Olin Energy, Sunderland, Great Britain) set at 20,700 kPa.

Analysis of plasma samples

To 0.5 ml of plasma were added 2.0 ml of chloroform and the mixture was agitated using a Vortex mixer for 30 sec. After centrifuging (3000 *g* for 10 min) to separate the layers, 1.5 ml of the chloroform phase was transferred to a tapered tube and evaporated to dryness in a stream of nitrogen. The residue was dissolved in 50 μl of the mobile phase under sonication and 20 μl was injected into the column. The above procedure was duplicated for each plasma sample. The Molsidomine peak height was measured and the plasma concentration calculated by reference to a standard curve obtained by processing control plasma containing known amounts of Molsidomine as described above. Peak height was plotted against plasma concentration over the range 0—10 ng/ml (human samples) and 0—10 μg/ml (dog samples). Plasma was stored at —20° until required for analysis.

RESULTS AND DISCUSSION

Recovery from aqueous systems

Aqueous buffer systems to which had been added Molsidomine (10 μg/ml) were extracted and analysed as described above for plasma (Table I). The percentage extracted was calculated by comparing the peak heights obtained following extraction of these buffered solutions with the peak heights of corresponding aqueous standard solutions. From pH 4 to 11 the recovery of the drug was virtually quantitative.

TABLE I

RECOVERY OF MOLSIDOMINE FROM AQUEOUS BUFFER SOLUTIONS

pH	Molsidomine extracted (%)
2.0	65
3.0	94
4.0	96
4.9	97
6.3	97
7.1	97
7.9	97
8.9	97
10.2	97
11.3	96
12.4	94

Recovery from plasma

Molsidomine was added to control plasma to give six replicate samples at five concentrations (2—10 ng/ml). This is the concentration range typically found in the plasma of human subjects following a therapeutic oral dose of 2 mg. The samples were processed as described and the recovery determined by referring the peak heights to a calibration curve obtained by analysing standard aqueous solutions of the drug (Table II). Single samples were analysed over a much wider concentration range (0.02—2.0 μg/ml) and the results indicate no significant dependence of recovery on concentration (Table II).

TABLE II

RECOVERY OF MOLSIDOMINE FROM PLASMA AT pH 7.4

C.V. = Coefficient of variation.

Molsidomine concn. (ng/ml)	Percent extracted ± C.V.	Molsidomine concn. (μg/ml)	Percent extracted
10	79± 6	2.0	87
8	79± 7	1.0	88
6	83± 5	0.5	88
4	85±24	0.2	84
2	85±14	0.1	85
		0.05	84
		0.02	83

Chromatography

Normal-phase chromatography on LiChrosorb SI 100 using 1,2-dichloroethane—methanol (95:5) as mobile phase was unsatisfactory, the Molsidomine peak eluting on the tail of a large plasma component [Fig. 1a]. Good separation of Molsidomine from the endogenous matrix was obtained on an octadecylsilyl reversed-phase column (Fig. 1b), eluting with an acetate buffer—methanol mobile phase.

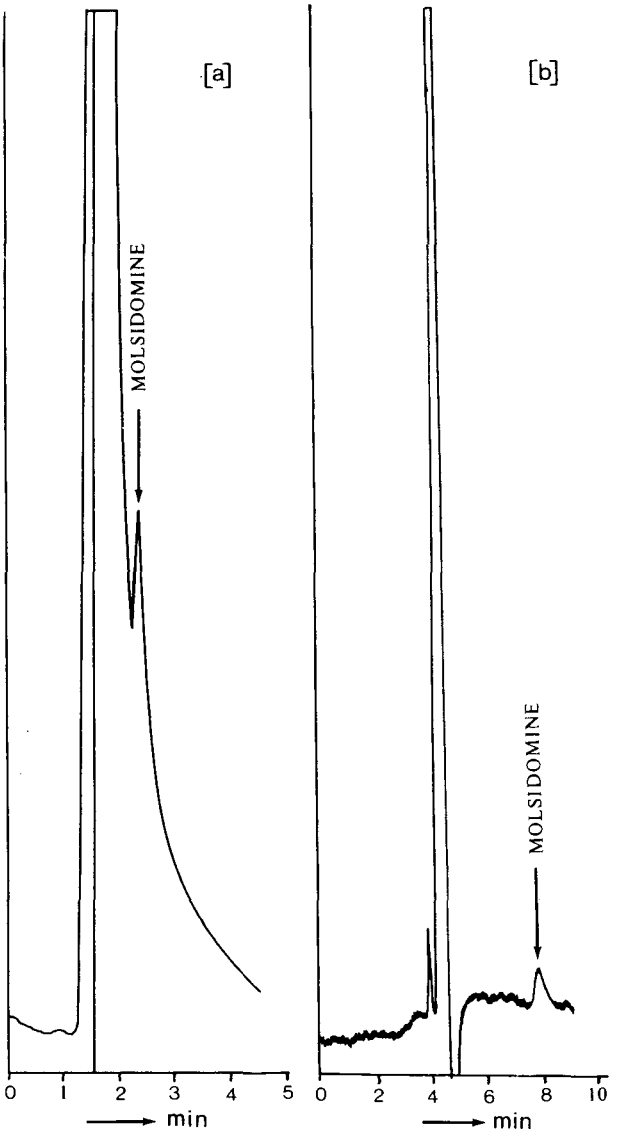


Fig. 1. Chromatogram of an extract of human control plasma to which had been added Molsidomine to give a plasma concentration of 10 ng/ml, a, LiChrosorb SI 100 column (0.005 a.u.f.s.); b, ODS Hypersil column (0.01 a.u.f.s.).

Limit of detection

A plasma concentration of 3.6 ng/ml gave a signal-to-noise ratio of 8 (Fig. 2). Extrapolating to a signal-to-noise ratio of 3 would yield a limit of detection of approximately 1.5 ng/ml. In practice, the precision of the method at a plasma concentration of 2 ng/ml (six replicates) was ± 16% (Table III).

Specificity

The specificity of the method was imparted by the chromatographic system and the ultraviolet detection wavelength. Evidence for the specificity was obtained from an experiment in which two beagle dogs were given an intravenous dose of 6 mg/kg of [^{14}C]Molsidomine. Blood samples were collected and total plasma radioactivity was determined by scintillation counting. Molsidomine concentrations were determined as described (Table IV). The peak corresponding to Molsidomine in the chromatogram was collected and the radioactivity content determined. Specific activity was obtained by dividing the micro-

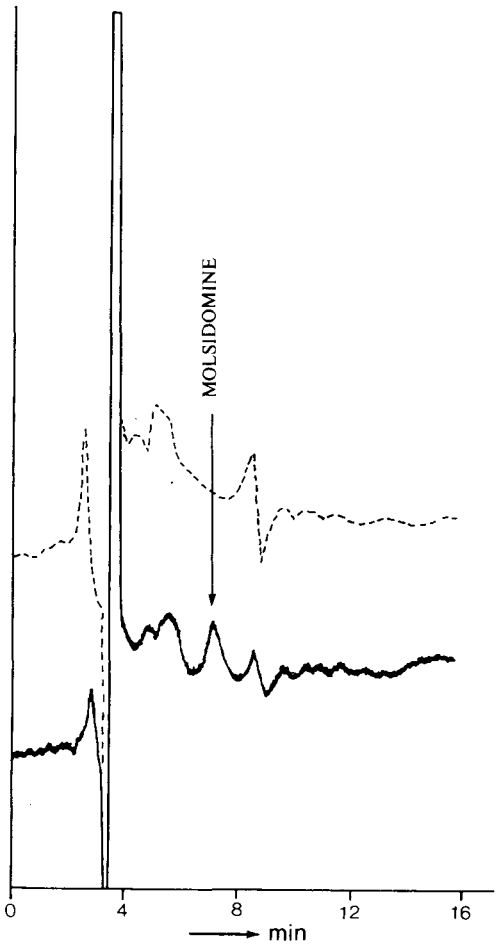


Fig. 2. Chromatogram of a plasma extract obtained from a human volunteer 45 min after receiving a p.o. dose of 2 mg of Molsidomine. The Molsidomine peak corresponds to a plasma concentration of 3.6 ng/ml (0.01 a.u.f.s.). - - - -, Control plasma extract; ——, plasma extract from volunteer receiving Molsidomine.

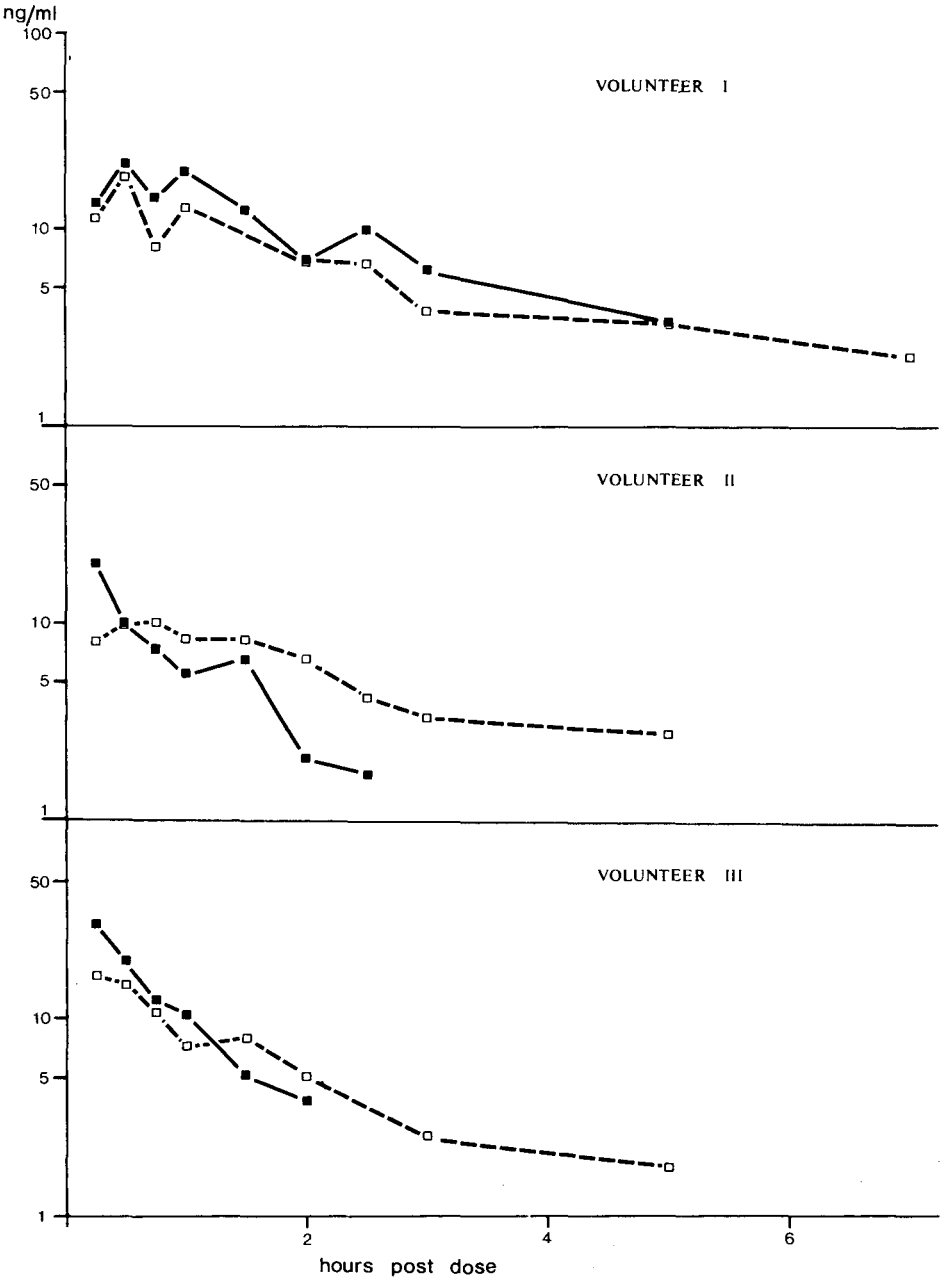


Fig. 3. The elimination of Molsidomine from the plasma of three human volunteers receiving a p.o. dose of 2 mg. ■——■, Tablet formulation; □- - -□, aqueous solution.

curies in the collected peak by the number of milligrammes of Molsidomine to which this peak corresponded. The mean specific activity for all samples was 0.65 ± 0.069 (10.6%) μCi/mg; this is in good agreement with the specific activity of the Molsidomine used in the experiment (0.60 μCi/mg). The total radioactivity in the plasma samples, in terms of the equivalent weight of Molsi-

TABLE III

PRECISION OF THE METHOD AND REGRESSION ANALYSIS (STANDARD CURVE)

s = standard deviation.

Molsidomine concn. (ng/ml)	Peak height (cm) at 0.01 a.u.f.s. deflection	Regression analysis		s	Precision (%)
		Fitted values	Departures (95% confidence)		
10.0	2.36 1.98 2.20 2.20 2.10 2.38	2.20	0.099	0.15	6.9
8.0	2.00 1.57 1.77 1.71 1.66 1.83	1.78	0.071	0.15	8.5
6.0	1.34 1.43 1.29 1.48 1.34	1.35	0.060	0.08	5.7
4.0	1.14 0.81 0.96 0.67 1.36 0.78	0.93	0.074	0.25	28.0
2.0	0.54 0.54 0.45 0.45 0.36	0.50	0.104	0.08	16.0

domine, was greater than the Molsidomine concentration as determined by the method, indicating the presence of metabolites in the plasma extract, which did not, however, interfere in the assay of Molsidomine.

Precision

This was evaluated from the data obtained from the samples used for the determination of recovery. The precision was calculated for each concentration (Table III). The calibration line was described by the regression equation: $y = 0.2132\ x + 0.0726$, where y is the Molsidomine peak height and x is the plasma concentration. The correlation coefficient was 0.985; this describes the "goodness of fit" of the experimental data with the regression line.

Calibration

Due to variations in response of the column and detector system, calibration

TABLE IV

MOLSIDOMINE AND ^{14}C CONCENTRATIONS IN DOG PLASMA FOLLOWING AN INTRAVENOUS DOSE OF 6 mg/kg

ND = not detectable (< ng/ml). For both dogs, mean specific activity = 0.65 (s = 0.069); specific activity of specific activity Molsidomine dosed = 0.60.

Time after dose	Dog A			Dog B		
	Molsidomine (μg/ml)	^{14}C (μg equiv. of Molsidomine per ml plasma)	Calc. specific activity (μCi/mg)	Molsidomine (μg/ml)	^{14}C (μg equiv. of Molsidomine per ml plasma)	Calc. specific activity (μCi/mg)
5 min	5.5	12.1	0.69	7.9	11.2	0.71
10 min	8.4	15.1	0.68	6.0	9.5	0.71
15 min	5.1	11.5	0.69	5.2	9.0	0.59
30 min	3.7	10.4	0.60	4.0	8.1	0.67
45 min	2.4	9.4	0.65	3.2	7.4	0.64
1 h	1.6	9.0	0.72	2.2	7.0	0.70
1.5 h	0.8	8.1	0.60	1.2	6.4	0.62
2 h	0.3	7.3	0.45	0.6	6.1	0.73
3 h	0.07	6.3	0.66	0.1	4.8	
4 h	ND	4.8		0.07	3.8	
6 h	ND	3.8		0.02	2.6	
24 h	ND	2.5		ND	2.0	
54 h		2.3		ND	1.9	
4 days		2.4			1.8	
8 days		1.6			1.0	

samples were always analysed on the same day as the unknown samples. The data presented in Tables II and III were obtained during one working day. Over a period of two weeks, during which time calibration samples were analysed daily, it was found that the variation of the slope of the standard curve about a mean line was ± 4%. The response of the column/detector system was linear from 2 ng to (at least) 2 μg injected.

Application

The method has been applied to the analysis of Molsidomine in the plasma of dogs receiving an intravenous dose of 6 mg/kg (Table IV), and to the analysis of the drug in human plasma following (a) a 2-mg p.o. dose of a tablet formulation, and (b) a 2-mg p.o. dose of an aqueous solution to three volunteers (Fig. 3).

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CHROMBIO. 215

QUANTITATION OF CHLORDIAZEPOXIDE AND ITS METABOLITES IN BIOLOGICAL FLUIDS BY THIN-LAYER CHROMATOGRAPHY

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SUMMARY

Chlordiazepoxide and its 4 major metabolites were assayed after separation by thin-layer chromatography following extraction from biological fluids. The compounds become intensely fluorescent in the presence of red, fuming nitric acid. The resulting compounds are quantitated with a spectrodensitometer with a fluorescent attachment. The sensitivity varies between 0.05 and 0.1 μg. The coefficient of variation is 1.4% for assays in urine and 6.4% in serum.

INTRODUCTION

Although the benzodiazepines have been studied extensively, many gaps still exist in our knowledge of these agents. As new benzodiazepines become available, research on the earlier compounds becomes somewhat neglected. Chlordiazepoxide (CDX) was the first widely used benzodiazepine and continues to be extensively prescribed; yet, information has only recently become available regarding plasma concentrations of the parent compound and several of its active metabolites after administration of "therapeutic" doses [1, 2]. The biotransformation of chlordiazepoxide has been reviewed by Schwartz [3]. Four major metabolites have been identified in man: desmethylchlor-

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diazepoxide (DM-CDX), demoxepam (DMX), desmethyldiazepam (DM-DZM) and oxazepam (OZM) (Fig. 1).

Spectrofluorometric and gas-chromatographic methods for the quantitation of CDX and its metabolites have been described [4—8]. These methods are not entirely satisfactory since they are often time consuming, do not assay all metabolites, are often affected by interfering substances, and may have inadequate sensitivity. Recently, Strojny et al. [9] reported a method for determination of CDX and three of its major metabolites by spectrophotodensitometry after thin-layer chromatographic (TLC) separation; a fourth metabolite of CDX, demoxepam, was determined by spectrofluorometry after selective extraction. We report here an accurate, precise and relatively simple method using quantitative TLC for the simultaneous assay of CDX and all four major metabolites in biological fluids.

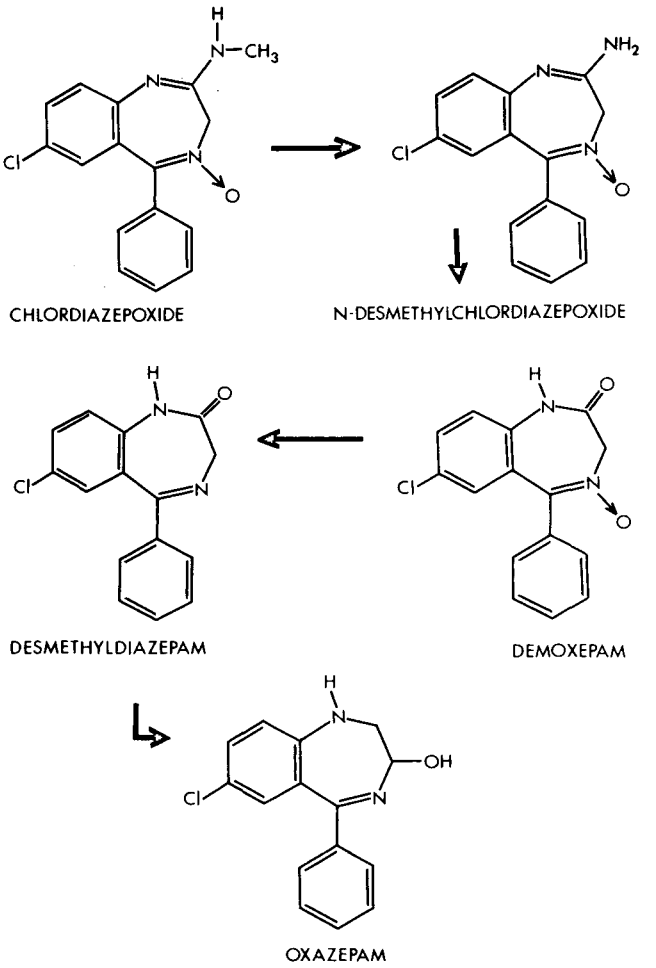

Fig. 1. Major routes of chlordiazepoxide biotransformation in man.

MATERIAL AND METHODS

Extraction from serum

Freshly drawn blood is rapidly centrifuged, preferably in the cold, and the serum stored at −20° until the assay is performed. A 2-ml volume of serum is placed in a 15 ml glass test tube and 0.5 μg prazepam in 5 μl methanol is added as an internal standard. Saturated sodium borate solution (4 ml; pH 9.4) is added to the serum and mixed well. The serum is then extracted three times with 4 ml diethyl ether. After each addition of ether the tube is shaken on a reciprocal shaker at low speed for 10 min, then centrifuged for 5 min in the cold to separate the two phases. The ether extracts are pooled and evaporated to dryness on a constant temperature block at 40° under a stream of nitrogen. The residue is quantitatively transferred to a 10 × 75 mm stoppered glass tube using small volumes of ether and again evaporated to dryness.

Extraction from urine

To assay for unconjugated drugs in the urine, saturated sodium borate solution (5 ml) is added to 10 ml urine and mixed well. Prazepam (0.5 μg) is also added to each urine aliquot. The urine is extracted three times with 5 ml diethyl ether and concentrated following the same procedure outlined above for serum.

Separation of the benzodiazepines

Quanta-Gram LQD, 20 × 20 cm TLC plates scored at 1-cm intervals (Quantum, Fairfield, N.J., U.S.A.) have proved to be very satisfactory for separation and subsequent quantitation of the benzodiazepines. It was not found advantageous to pre-activate these plates. It is not advisable to use the first and last channels of the plates as they tend to run less uniformly than the other channels.

The residue from the ether extraction is dissolved in 100 μl absolute methanol and a 50-μl sample is applied to the spotting area of the TLC plate. A mixture of CDX and its metabolites for use as standards is prepared by dissolving 0.1 mg of each in 1.0 ml absolute methanol. Blank serum or urine is spiked with 0.2, 0.4, 0.8, 1.0 and 2.0 μg of each of the five benzodiazepines to be assayed along with 0.5 μg prazepam. The benzodiazepines are then extracted, redissolved in methanol, and applied to individual channels on the same TLC plate as the unknowns. Fresh standard solutions should be prepared at least every 4 weeks, stored at - 20° and protected from light. Standdards should be run on every plate, as no two plates behave identically. The spots are dried in a stream of nitrogen after application to the plate.

Of the many developing solvent mixtures examined, the following was found to give good separation of CDX, its four major metabolites, and prazepam, the internal standard: acetone—dioxane—isopropanol—*n*-heptane—toluene—hexane (15:15:30:30:30:25, v/v) at 22°. Solvents of nanograde quality were used whenever available. The developing mixture must be prepared fresh daily in order to obtain reproducible separation of the benzodiazepines. The TLC tank is lined with filter paper saturated with the solvent mixture

TABLE I

R_F VALUES FOR CHLORDIAZEPOXIDE AND 4 OF ITS METABOLITES, AND FOR PRAZEPAM

Substance	R_F
Prazepam*	0.64
Desmethyldiazepam	0.58
Chlordiazepoxide	0.48
Demoxepam	0.40
Desmethylchlordiazepoxide	0.31
Oxazepam	0.23

*Internal standard.

and the system allowed to equilibrate for 30 min. After placing the TLC plate in the tank, the solvent front is allowed to migrate 16 cm from the baseline (50—60 min). This system separated the areas of maximum fluorescence of each of the compounds by at least 1 cm. Representative R_F values obtained are listed in Table I.

Quantitation of the chromatogram

When exposed to short wave-length ultra-violet light, the benzodiazepines fluoresced to a slight and variable extent. The plate is placed for 45—50 min in a glass tank containing a small beaker of red, fuming nitric acid. This procedure increases the fluorescence of the spots considerably. The plate is removed from the tank, left under a fume hood for 30 min, and then placed in an oven at 100° for 30 min. The latter further intensifies the fluorescence. After this treatment the fluorescence is stable for at least one week.

To quantitate the benzodiazepines, the plate is then scanned with a Schoeffel SD 3000 spectrodensitometer with SDA 335 fluorescence attachment, coupled to a Schoeffel SDC 300 density computer (Schoeffel, Westwood, N.J., U.S.A.). The light source is passed through a Corning No. 271 filter (Corning, Corning, N.Y., U.S.A.). Maximal fluorescence is obtained with the reflectance mode monochrometer of the SDA unit set at 680 nm. The slit width of the exciting beam is 1 mm and the plate speed 2 in. per min. A black line scored across the chromatogram at the junction of the spotting area and the silica gel provides a useful reference point. This line appears on the chart record as a negative deflection. The distance from this point to the peaks is used to calculate R_F values as a check on the identity of the benzodiazepines. One of the unused lateral channels on the TLC plate may be used to set the baseline for the recorder. For each benzodiazepine a standard curve is constructed from a linear-linear plot of the benzodiazepine—prazepam peak height ratio for each benzodiazepine versus concentration. The peak height ratio of each benzodiazepine in the unknown is compared to its respective standard curve for quantitation.

RESULTS

Recovery of each of the 5 compounds added to blank serum or urine is 80% or greater. A 0.5-μg sample of each of the 5 compounds per 10 ml urine or per 2 ml serum can be assayed with a coefficient of variance between 1.4 and 6.4%. The standard curves for all 5 compounds are linear from at least 0.1 to 2.0 μg (Fig. 2). DM-DZM and DM-CDX can be measured in amounts as low as 0.05 μg. CDX, DMX and OZM are easily quantitated in amounts as low as 0.1 μg.

Attempts were made to hydrolyze the glucuronide conjugates by acid hydrolysis and by addition of large amounts of β-glucuronidase. In either case, the conditions required resulted in some degradation of the benzodiazepines. As a result, the distinct separation after extraction of the unconjugated compounds could no longer be attained.

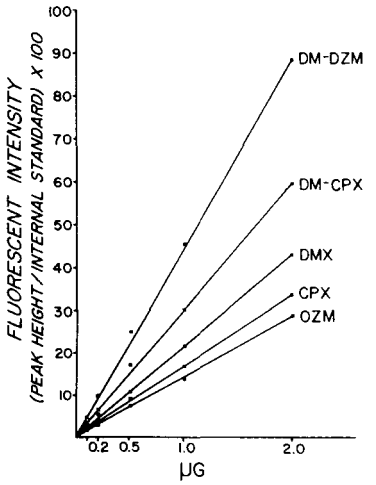

Fig. 2. Standard curves for chlordiazepoxide and each of its major metabolites.

Interfering substances

In order to investigate the possibility of interference produced by drugs (some of which, patients might take concomitantly with CDX) a wide spectrum of drugs was tested in the TLC system (Table II). The specified quantity of each drug was added to blank plasma alone and also with 1.0 μg of each of the 5 benzodiazepines. A band of absorbance (char) appeared just above the fluorescent band of the internal standard, prazepam, in all serum samples analyzed. This band was presumably due to lipids and did not interfere with the fluorescence of prazepam.

Serum levels after single and multiple dose chloridiazepoxide administration

The plasma-level—time relationship of CDX and its 4 metabolites after the administration of a single 50 mg dose of Librium® to a 50 kg female

TABLE II

INTERFERENCE OF DRUGS THAT MIGHT BE ADMINISTERED CONCOMITANTLY WITH CHLORDIAZEPOXIDE

1 = No discernible fluorescence; 2 = absorbance (char), no interference with fluorescence of benzodiazepines

Drug	R_F value	Amount applied (μg)	Interference
Clonazepam	—	2	1
Diazepam	0.59	2	Moderate fluorescence, poor separation from desmethyldiazepam
Flurazepam	—	2	1
Flunitrazepam	—	2	1
Nitrazepam	—	2	1
Temazepam	0.49	2	Strongly fluorescent band overwhelms CDX in this amount
Chlorpromazine	0.13	2	2
Thioridazine	—	2	1
Glutethimide	—	5	1
Meprobamate	—	1.6	1
Amitriptyline	—	2	1
Desipramine	—	2	Narrow band of absorbance just above baseline
Imipramine	0.10	2	2
Nortriptyline	—	2	1
Furosemide	—	1.6	1
d-Propoxyphene	—	2	1
Aspirin	—	2	1
Salicylic acid	—	20	2
Carbamazepine	0.48	5	Absorbance, quenches chlordiazepoxide fluorescence
Phenobarbital	—	40	1
Phenytoin	—	20	1
Quinidine SO_4	—	2	Strongly fluorescent band just above baseline

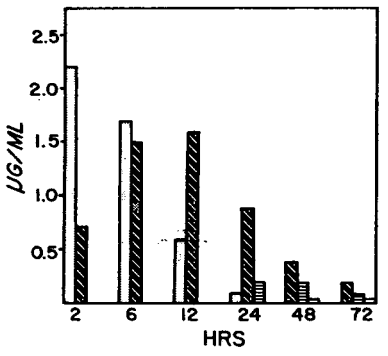

Fig. 3. The plasma concentration of chlordiazepoxide and four metabolites at varying times after administration of a single 50-mg dose of Librium orally (50 kg female). ▨ , CDX; ▧ , DM-CDX; ▤ , DMX; □ , DM-DZM.

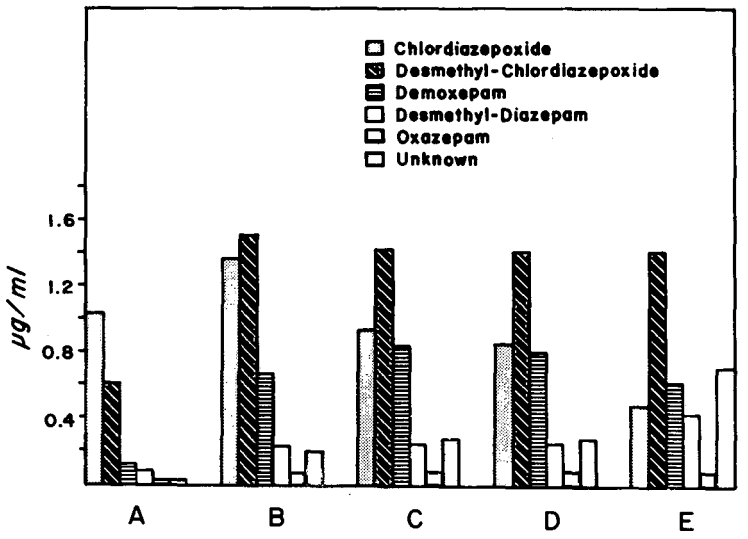

Fig. 4. Plasma concentrations of chlordiazepoxide and four metabolites at varying times after administration of 25 mg Librium every 8 h for 4 days (86 kg male). A = 24 h after first dose; B = 96 h after first dose; C = 12 h after final dose; D = 18 h after final dose; E = 36 h after final dose.

volunteer is shown in Fig. 3. The plasma levels of the 5 benzodiazepines during and after administration of 25 mg of Librium, 3 times a day for 4 days to an 86 kg male volunteer are shown in Fig. 4.

DISCUSSION

Studies on the pharmacokinetics of CDX and its metabolites have been hampered by the lack of specific, sensitive, accurate and rapid assays. We report here an assay which conforms to three of these requirements. Although the assay takes several hours to complete, the method is easily performed and allows 12 samples of blood or urine to be assayed for CDX and 4 metabolites simultaneously, on a single TLC plate. In addition, many other drugs which might be administered concommitantly with CDX do not interfere with the assay for CDX or its metabolites. In our method, elution of the compounds from the TLC plate for quantitation by other means is not required. In contrast to the TLC method of Strojny et al. [9], our method requires neither multiple developments in order to separate lipid materials from the benzodiazepines nor a separate assay method for determination of demoxepam. The precision of quantitation is increased by addition of an internal standard, prazepam. This method can be adapted to the determination of other benzodiazepines by selecting the appropriate developing solvent system.

The use of the borate solution with a pH of 9.4 and gentle shaking of the aqueous ether mixture makes a considerable contribution to ease of extraction by diminishing emulsion formation. The duration of exposure of the chromatogram to the red fuming nitric acid is critical. Excessive exposure

will eventually reduce fluorescence to zero. Similarly, prolonged heating will reduce fluorescence.

Dixon et al. [10] reported that DM-DZM is a metabolite of CDX in man. These investigators separated DM-DZM from the other metabolites by TLC. However, their system does not satisfactorily separate CDX and its other metabolites from each other.

The TLC method we report here can quantitate the four major metabolites in the serum of individuals receiving 25 mg CDX chronically three times a day. Following a single dose, however, the level of OZM, which is at the distal end of the biotransformation pathway and has the shortest elimination half-life of the various metabolites, is detectable but its level is too low for accurate quantitation. An unknown metabolite also appears with chronic dosing (Fig. 4). Studies to identify this metabolite are in progress.

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Biomedical Applications

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Note

Rapid and simplified extraction procedure for gas chromatographic—mass spectrometric profiling of urinary organic acids

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The application of gas chromatography (GC) and combined gas chromatography—mass spectroscopy (GC—MS) for the profiling of urinary organic acid metabolites has led to the detection of several new inborn errors of metabolism [1]. The isolation of the organic acids from urine prior to GC is usually done either by anion-exchange chromatography or by solvent extraction techniques. Because ion-exchange methods are more laborious and time consuming, solvent extraction is normally preferred for metabolite screening. We now describe a more rapid and simplified solid-phase extraction procedure for urinary organic acids.

EXPERIMENTAL

Materials and methods

Solid phase extraction tubes (Jetubes), of 3-ml capacity, were purchased from Manhattan Instr. (Santa Monica, Calif., U.S.A.). J.T. Baker (Phillipsburgh, N.J., U.S.A.) Analyzed Reagent Grade solvents were used.

Urine extraction methods. Urine from a 30-year old male was used to compare the manual and solid phase isolation methods. A first morning sample (creatinine level 311 mg/dl) was collected. Five aliquots, 3 ml each, of the urine were pipetted into test tubes. To each tube was added the internal standard, 3-chlorophenylacetic acid (0.212 mg) and hydroxylamine hydrochloride

(30 mg) and the pH was adjusted to 12 with 2 *N* NaOH. Each sample was heated at 60° for 30 min to form the oximes of the keto acids. After cooling to room temperature, the samples were divided into two sets of 5, one of which was extracted by the solid phase method and the other by the conventional manual extraction method.

Solid phase extraction. One and one-half of each aliquot was acidified to a pH below 3 with concentrated HCl and transferred directly onto a solid phase extraction tube. The organic acids were eluted with a mixture of diethyl ether—ethyl acetate (1:1, 24 ml). The extract was dried ($MgSO_4$), evaporated and transferred to a vial with ethyl acetate—methanol (1:1, 0.5 ml). The solvent was removed with a stream of nitrogen and derivatized as described below.

Manual extractions. One and one-half ml of each aliquot was acidified to a pH below 3 with concentrated HCl, and the organic acids were extracted with three 8-ml portions of diethyl ether—ethyl acetate (1:1). The combined extracts were washed and dried ($MgSO_4$) and evaporated to dryness. The extract was transferred to a vial with ethyl acetate—methanol (1:1, 0.5 ml). The solvent was removed with a stream of nitrogen and derivatized as described below.

Preparation of derivatives

The residues from above were derivatized with bis(trimethylsilyl)trifluoroacetamide (50 μl) at 60° for 30 min. Between 1 and 2 μl of this solution was used for GC—MS of the sample.

GC—MS analysis. GC and GC—MS experiments were performed on a Finnigan Model 9500 gas chromatograph, employing a 6-ft. U-shaped 1/8-in. I.D. column, packed with 10% OV-17 on 100—120 mesh Gas-Chrom Q. Mass spectra were recorded with a Finnigan model 1015 quadrupole mass spectrometer controlled by a Digital Equipment Corp. (DEC) PDP-11/20 computer. Subsequent data processing was done on a DEC PDP-11/45 with 28K words of core memory, a 5M word disc drive, teletype, printer, CRT, and Versatek printer/plotter. Each analysis was carried out by co-injecting 1—2 μl of the sample with a solution of three straight-chain hydrocarbons (with 12, 18 and 24 carbon atoms) in ethylbenzene (1 μg of each in 0.2 μl) at 70°. After 4 min The column was programmed at 4°/min and the column effluent was introduced into the MS instrument. A total of 600 mass spectra were recorded to a final temperature of 285°.

Data processing. The procedures for analyzing the raw data of the GC—MS computer system have been described previously [2]. The HISLIB program [2] was used to examine statistically the sets of GC—MS profiles collected for each extraction procedure.

RESULTS AND DISCUSSION

Solid-phase extraction tubes, which were originally developed at the Jet Propulsion Laboratory for use in the National Aeronautics and Space Administration program, are polypropylene tubes filled with an inert cellulose gauze matrix that has been purified by extensive solvent treatment [3]. The hydrophilic nature of the solid phase allows a liquid—liquid extraction separation of aqueous polar compounds from hydrophobic acids and neutrals. Advantages of

the method are the elimination of problems associated with emulsions and tube transfers. A typical urine extraction takes about 5 min whilst a comparable manual extraction takes 30 min. Since the procedure is greatly simplified, the use of a smaller starting volume (less than 0.5 ml) is possible. This is of considerable importance because urine collection from newborn babies is difficult and a number of analyses may be necessary to confirm an inborn error of metabolism.

By running 5 duplicate experiments by solid-phase and manual-extraction methods, we have established that the new technique gives comparable recoveries of the organic acids in the samples (Table I). As can be seen from the re-

TABLE I

COMPARISON OF RELATIVE CONCENTRATIONS OF ORGANIC ACIDS DETECTED AND THEIR PERCENT STANDARD DEVIATION

Acid	Manual extraction			Solid-phase extraction	
	RRI*	Relative concn.**	Percent standard deviation (n=5)	Relative concn.**	Percent standard deviation (n=5)
Glycolic	1131	42.3	21.4	64.4	15.6
3-Hydroxyisobutyric	1206	27.8	6.5	26.8	6.7
Pyruvic	1224	14.0	17.6	13.0	6.6
0-Cresol	1235	53.0	24.6	32.7	16.2
3-Hydroxyisovaleric	1244	9.0	18.7	15.4	33.9
Urea	1369	176.8	72.3	374.5	19.9
Methylfumaric	1472	11.3	24.1	13.6	15.1
Adipic	1606	8.1	11.6	7.5	20.5
Pyroglutamic	1679	28.2	30.3	38.6	20.2
5-Hydroxymethyl-furoic	1690	140.1	4.5	161.9	18.8
2-Ketoglutaric	1728	—	—	39.3	9.6
3-Hydroxyphenyl-acetic	1735	44.7	11.8	46.6	26.5
4-Hydroxyphenyl-acetic	1766	65.8	10.3	80.4	15.4
2,5-Furandicarboxylic	1806	145.0	6.4	186.2	12.9
trans-Aconitic	1858	59.4	16.5	65.7	10.2
Citric	1888	195.8	24.3	212.0	18.8
3-(3-Hydroxyphenyl)-hydracrylic	1943	269.3	9.2	321.4	20.6
Hippuric***	2118	945.3	27.3	1614.0	19.7
3-Indoleacetic	2191	13.0	33.9	28.5	22.5
Stearic	2288	6.6	21.3	—	—
3-Hydroxyhippuric	2380	320.2	9.4	389.1	12.7
4-Hydroxyhippuric	2459	23.4	11.5	32.2	15.5

*Relative retention index (on n-alkane scale).

**The internal standard, 3-chlorophenylacetic acid, has its concentration set at 100. All quantitative results are expressed in relative concentration units. Relative concentration values have not been corrected for differential extraction or detection coefficients.

***Hippuric acid elutes as a mixture of mono- and di(trimethylsilyl) derivatives. The relative concentrations of the two peaks have been combined and are reported as the mono(trimethylsilyl) derivative.

sults most metabolites are extracted with comparable efficiencies and reproducibility, and some acidic metabolites are extracted more efficiently by the solid-phase extraction method. Because elution of the extraction tube with ether—ethyl acetate could potentially add contaminants (plasticizers or nonpolar residues) from the solid phase matrix, a solvent blank was run through the tube and analyzed. When this sample was run through GC—MS and data analysis, no interfering substances were found to be present.

We conclude that solid phase extraction of organic acids from urine is simpler, faster, and less laborious than manual extraction with the same solvents. We find that most acidic metabolites are extracted with equal or improved efficiencies.

A subsequent publication will describe in detail the experimental procedures and the data processing techniques used for the analysis of organic acids.

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Note

New method for quantitative analysis of pyridoxal-5′-phosphate in biological material

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The chemical methods in use for determination of pyridoxal-5′-phosphate (PLP) in biological samples are all based on initial rate studies of enzyme-catalyzed reactions where PLP is necessary as a co-factor. By using well-resolved apoenzyme preparations and proper reaction conditions it is possible to determine quite low PLP concentrations from the linear dependence usually obtained between reaction rate and PLP concentration. In the most commonly used method, the rate of $^{14}CO_2$ formation from (L)-(-)-tyrosine-$^{14}C_1$ under the influence of PLP-stimulated tyrosine apodecarboxylase is estimated by trapping of the liberated $^{14}CO_2$ and liquid scintillation counting [1—5]. A method recently published by Suelter et al. [6] makes use of apotryptophanase and UV determination of liberated *o*-nitrothiophenolate from a synthetic chromogenic substrate, S-*o*-nitrophenyl-L-cysteine.

In connection with investigations concerning the purification of tyrosine apodecarboxylase (TAD) from *Streptococcus faecalis* [7], a need arose for a simpler and more reliable method for enzyme activity determinations. Because the enzyme was known to exhibit activity towards L-3,4-dihydroxyphenylalanine (L-DOPA) [8], a quantification of the dopamine produced by the reaction by means of chromatographic separation and amperometric detection (LCEC) [9], was considered as favourable in view of the extreme sensitivity of

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the electrochemical detector [10,11]. In this paper we present some results which clearly demonstrate certain advantages of this method over the previously used radioenzymatic one.

MATERIALS AND METHODS

Conditions for the enzymatic decarboxylation

Enzyme preparation. In all experiments the reaction mixture was completely homogeneous. For this purpose a TAD preparation, obtained using the prepurification procedure previously described [7] for the commercially obtained cell material (No. T 4629; Sigma, St. Louis, Mo., U.S.A.) was used throughout. This apoenzyme was shown to possess very little decarboxylase activity in the absence of any added PLP, indicative of the high resolution achieved.

Sample preparation. The standard curves were generated by the use of PLP-solutions obtained by dilutions (1:4 to 1:16) of a common stock solution containing 96 ng/ml. Plasma (1.5 ml) was deproteinized with 75% trichloracetic acid (150 μl) and centrifuged. A 700-μl sample of the supernatant was diluted with 800 μl of 0.1 *M* sodium acetate buffer pH 5.5 and 1.0 ml was diluted to 2 ml with 1 *M* acetate buffer pH 6.5. The PLP standards were treated similarly.

Kinetic procedure. The reaction was carried out in small, stoppered centrifuge tubes at 30.0°. Each tube was first supplied with 700 μl of the TAD-preparation and 300 μl of the PLP-containing sample. After 1 h of pre-incubation, 400 μl of a 4 m*M* solution of L-DOPA in 0.1 *M* acetate buffer was quickly added and a chronometer started. At the time t, 200 μl of the reaction mixture was rapidly quenched with 800 μl of 0.3 *M* perchloric acid. After centrifugation this solution was then subjected to LCEC analysis. If the analysis was not carried out immediately, the acid quenching solution was permitted to contain a small amount of antioxidant such as bisulphite or mercaptoethanol.

Chromatographic procedure

Instrumentation. The LCEC equipment was constructed from an Altex Model 100 constant-flow solvent pump, a Rheodyne Model 7120 injection valve provided with a 20 μl loop, an Altex 250 × 4.6 mm stainless-steel column, slurry-packed with 10 μ Nucleosil SA, a spherical, surface-porous cation exchanger, an electrochemical detector cell packed with silicone-oil-based graphite paste and equipped with a 50 μ PTFE spacer, a reference electrode compartment, an operational amplifier capable of converting 1nA to 1V and a Linear Model 264 potentiometric recorder. The detector and amplifier parts were obtained from Bioanalytical Systems (West Lafayette, Ind., U.S.A.). The column was separated from the pump by means of a 5 m × 1/16 in. O.D. PTFE coil and the potentiostat was operated at 0.55 V vs. the Ag—AgCl-reference electrode. The column, detector and amplifier were all contained within a Faraday-cage and carefully grounded in order to minimize electrical noise [11].

Chromatographic conditions. A citrate—acetate buffer of pH 5.2 was used throughout. This was prepared from 23.0 g of citric acid, 16.6 g of anhydrous sodium acetate, 4.20 ml of acetic acid and 9.6 g of sodium hydroxide dissolved in 4000 ml of permanganate-distilled water. During chromatography the buffer was heated to 45° in order to avoid air-bubble formation in the detector

cell. Flow-rate was set at 0.60 ml/min in all cases, unless otherwise stated.

Chemicals. All chemicals used were of analytical grade quality. The buffer chemicals were obtained from Merck (Darmstadt, G.F.R.) and pyridoxal-5'-phosphate and 3,4-dihydroxyphenylalanine from Sigma (St. Louis, Mo., USA).

RESULTS AND DISCUSSION

The chromatographic result from a typical kinetic run in which a plasma sample provided the PLP source is shown in Fig. 1. When the dopamine (DA) peak heights were plotted against the reaction time a perfectly straight regression line was obtained (Fig. 2.). The least-squares-fit showed a correlation coefficient of 0.9980. From Fig. 1 it is quite evident that the corresponding loss in L-DOPA concentration is less than 5%, which means that the reaction is essentially of pseudo-zero order.

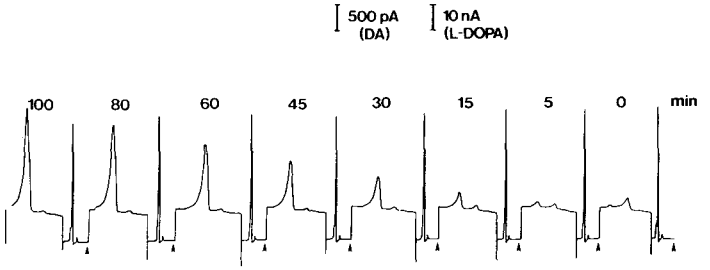

Fig. 1. Chromatographic recordings showing the progress of DA (left) formation in a plasma PLP-stimulated enzymatic decarboxylation of L-DOPA (right). The small, middle peak of each chromatogram corresponds to a hitherto unidentified component from the plasma.

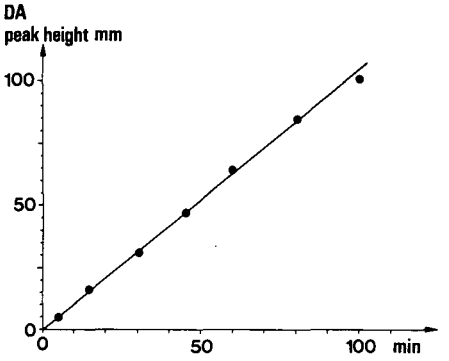

Fig. 2. Linearity obtained by graphic evaluation of the results shown in Fig. 1, illustrating the precision of the method for the initial rate determinations.

In another run the reactions were quenched at a single reaction time of 45 min (Fig. 3). A plot of the DA peak height obtained against the concentration of the standard PLP solutions used, gave a linear standard curve. Linear regression by the least squares method gave a correlation coefficient of 0.9992. This standard curve covers the normal range of plasma PLP which has been found by earlier investigators [1,3] to be within 5—20 ng/ml. The relative standard

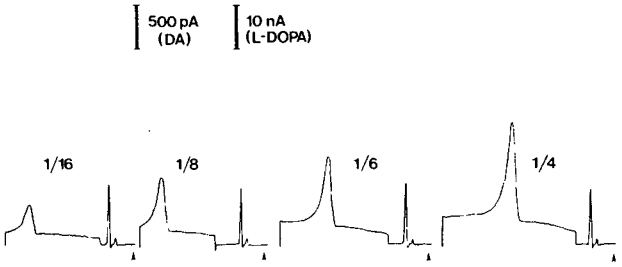

Fig. 3. Chromatographic recordings showing the relative amounts of DA formed at different PLP concentrations after a fixed reaction time of 45 min. Reaction conditions were otherwise identical.

deviation obtained upon repeated analyses of PLP from the same plasma pool with the use of a single reaction time (45 min) amounts to 6%. The recovery of PLP added to plasma was not investigated, because it has been shown earlier [1,3] to be ca. 90%.

Under the conditions used the retention volumes for L-DOPA and DA are 4.8 ml and 10.9 ml, respectively, which means that at a flow-rate of 0.60 ml/min one chromatogram will require ca. 20 min. We have found, however, that the flow-rate can be increased considerably, to speed up the analysis, with no other disadvantage than the accompanying rise in pressure.

It should be emphasized that because of the very small amounts required for each injection and the dilution made upon the quenching, all volumes described in the sample preparation and kinetic procedures can be scaled down considerably to suit the particular needs of a micro-method.

The sensitivity of the method, however, is highly dependent upon the quality of the apoenzyme. In our investigation we have found that under the conditions used, an over-all volume reduction will permit the analysis of 100 μl plasma, i.e. the method is sensitive enough for 1 ng of PLP and even less.

CONCLUSION

The use of high-performance liquid chromatography with LCEC for the monitoring of PLP-dependent, enzymatic decarboxylation of L-DOPA to DA, has been shown to provide an excellent method for the quantitative determination of PLP in biological fluids, such as plasma or serum. The method makes use of resolved and partially purified tyrosine apodecarboxylase (E.C. 4.1.1.25) from *Streptococcus faecalis*, a PLP enzyme which is sufficiently active towards L-DOPA as a substrate to permit initial rate determinations to be carried out with high precision, even at very low PLP concentrations. Because of the great selectivity and sensitivity inherent in the LCEC procedure, the reaction product, DA, can be quantitated in very low amounts, a fact which obviates the earlier need of a radiolabelled substrate. A further advantage of the method over the previously used radioenzymatic procedure is found in the very easy handling and control of the reaction mixture prior to analysis, which permits samples to be withdrawn and quenched at very precise time intervals, a prerequisite for accurate kinetic studies.

Our results have shown that this method for PLP determination is reliable, uncomplicated and easy to perform with a high degree of precision. In our opinion it is in many respects superior to the radioenzymatic procedure. It may also be suggested that the LCEC technique should be a very valuable tool for the study of selected enzyme-catalyzed steps in the area of tyrosine as well as tryptophan metabolism. Work in this field has recently been reported [12] and is also in progress in our laboratory.

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Note

Determination of histamine in plasma by high-speed liquid chromatography

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The fluorometric method so far described for determination of histamine in biological samples is based on the reaction of histamine with *ortho*-phthalic aldehyde (OPT) in an alkaline medium to form a fluorescent product, which is converted by acidification to a more highly fluorescent and stable species [1]. Although fluorescent products formed from many amines of biological importance, with OPT in alkaline media, are destroyed by the acidification, spermidine and a few amino acids give acid-stable fluorescent products with the same procedure [2—4]. Therefore, for the assay of histamine in biological samples by the OPT method, a special purification technique such as ion-exchange chromatography has been required prior to reaction with OPT [5—9].

Recently, we found that the fluorophores of histamine and spermidine with OPT could be separated successfully by reversed-phase high-speed liquid chromatography (HSLC) though their structures remained unknown. In this paper, HSLC is used effectively for the determination of histamine in plasma. This method gives reliable results and is readily performed with 1 ml of plasma containing as little as 0.2 pmoles of histamine.

EXPERIMENTAL

Chromatographic apparatus, and preparation of the column and the mobile phase

The liquid-chromatographic system consisted of a Hitachi 635 Liquid Chromatograph, a Hitachi 203 Spectrophotofluorometer equipped with a Hitachi flow-cell unit (cell volume, 20 μl) and a Hitachi 056 Recorder (chart speed, 2.5 mm/min). The fluorescence intensity was monitored at the emission wavelength of 450 nm with the excitation wavelength set at 350 nm.

A stainless-steel column (15 × 0.4 cm, I.D.) was packed by the slurry tech-

nique: to 10 ml of a mixture of dioxane, tetrachloromethane and tetrabromoethane (40:40:20), 1.0 g of LiChrosorb RP-18 (5 μm; Merck, Tokyo, Japan) was added. After the slurry was poured into the packing reservoir, chloroform was made to flow through the column at 150—300 kg/cm^2 for 1 h. Then, the mobile phase described below was made to flow at the rate of 0.5 ml/min for 30 min. The column thus packed can be used for more than 300 injections with only a small decrease in the theoretical plate number. The system was operated at room temperature.

The mobile phase was a mixture of 0.2 *M* NaCl solution and methanol (55:45) adjusted to pH 3 with 0.1 *M* HCl, by measurement with a Hitachi-Horiba M-7 pH meter.

Chemicals and reagents

All of the chemicals and solvents used were of reagent grade. *n*-Butanol, benzene and methanol were redistilled before use. An NaCl-saturated sodium hydroxide solution was prepared by adding 30 g of NaCl to 100 ml of 1 *M* NaOH.

Standard solution

A stock solution (1×10^{-3} *M* histamine) was prepared by dissolving 184 mg of histamine·2HCl (Wako, Osaka, Japan) in 100 ml of distilled water. The working solution (5×10^{-8} *M* histamine) was prepared by diluting the stock solution with distilled water to use within a day.

Analysis of plasma

To 1.0 ml of plasma placed in a 10-ml centrifuge tube, 1.0 ml of 0.4 *M* $HClO_4$ was added. The mixture was stirred well and centrifuged at 1200 *g* for 10 min. A 1.0-ml sample of the supernatant solution was transferred to a 10-ml glass-stoppered shaking tube containing 0.3 ml of 5 *M* NaOH, 0.4 g of NaCl and 2.5 ml of *n*-butanol. The contents of the tube were shaken for 5 min on a mechanical shaker. After centrifugation, the aqueous phase was aspirated, followed by the addition of 1.5 ml of NaCl-saturated sodium hydroxide solution to remove contaminants such as amino acids. The contents of the tube were again mixed for 3 min. After centrifugation, 2.0 ml of the organic phase were transferred to another 10-ml glass-stoppered shaking tube containing 0.25 ml of 0.1 *M* HCl and 2.0 ml of benzene. The mixture was shaken for 3 min. After centrifugation, 0.1 ml of the acidic aqueous phase was transferred to a small test-tube, to which 20 μl of 1 *M* NaOH and 5 μl of 1% OPT—methanol solution were successively added to develop fluorescence. After mixing for exactly 4 min, 10 μl of 0.95 *M* H_2SO_4 was added to stop the fluorescence reaction. A 100-μl volume of the mixture was injected for HSLC—fluorescence analysis. As the standard, 1.0 ml of histamine working solution described above was used instead of plasma. Concentration of plasma histamine was calculated as follows:

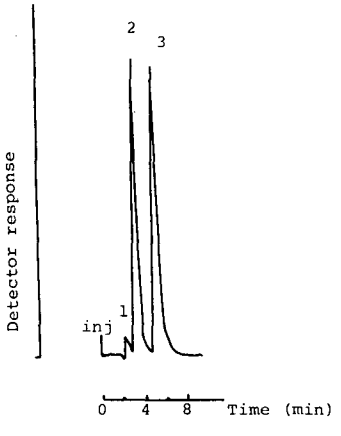
$$\text{Histamine concentration (ng/ml)} = \frac{111}{1000} \times \frac{\text{peak height of sample (mm)}}{\text{peak height of standard (mm)}} \times \left[\begin{matrix}\text{concentration of histamine} \\ \text{standard solution (pmole/ml)}\end{matrix}\right]$$

where $C_5H_9N_3 = 111$.

RESULTS AND DISCUSSION

As shown in Fig. 1, OPT-histamine fluorophore (OPT-his) and OPT-spermidine fluorophore (OPT-spd) formed in the reaction mixture were completely separated, with retention times of 5.0 and 3.0 min, respectively. The peaks observed at the retention time of 2.0—3.0 min were due to the reagent blank. Since the reagent blank showed a weak fluorescence, overlapped with the fluoroscence of OPT-his in the range of 400—480 nm, the separation of OPT-his from the fluorophore of the reagent blank greatly enhanced the sensitivity of the method for the determination of histamine by OPT reagent.

When the methanol content of the mobile phase decreased, the retention time of OPT-his was delayed considerably with broadening of the peak, though the peaks of OPT-spd and the reagent blank remained unchanged. On the other hand, if the methanol content was more than 60%, the peaks of OPT-his and OPT-spd became overlapped. The pH of the mobile phase and the presence of NaCl in the mobile phase were found to affect the peak width of OPT-his. When the pH was made greater than 3 and NaCl was not added, the peak of OPT-his tailed badly.



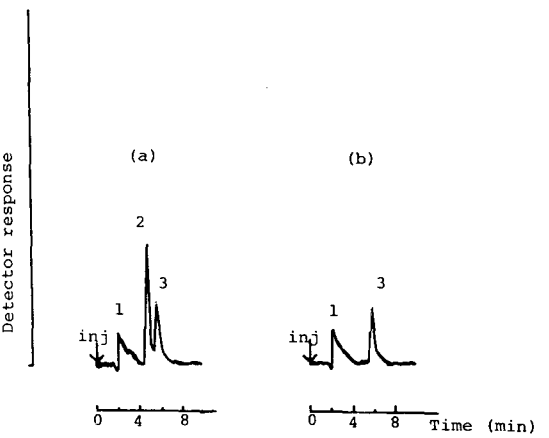


Fig. 1. Separation of OPT-his and OPT-spd. A mixture of 1×10^{-7} *M* histamine and 1×10^{-4} *M* spermidine was used. Peaks: 1, OPT reagent blank; 2, spermidine; 3, histamine.

Fig. 2. Chromatograms from analysis of plasma sample under the prescribed conditions. (a) = plasma (0.84 ng/ml histamine); (b) = reagent blank. Peaks: 1, OPT reagent blank; 2, histamine; 3, unidentified peak caused by the extracting solvents.

Fig. 2 shows typical chromatograms obtained with plasma and the reagent blank under the given conditions. The peak observed at the retention time of 6.0 min was caused by the extracting solvents.

There was a linear relationship between the fluorescence intensity (peak height) and the concentration of histamine in the range of 0.2—100 pmole/ml. The histamine content of plasma might be calculated by the ratio of the peak height of sample to that of a standard solution. The limit of sensitivity of the method was 0.2 pmoles of histamine, which corresponded to 22.2 pg/

ml when calculated in terms of sensitivity for plasma. The sensitivity was defined as the amount giving a signal-to-noise ratio of 2.

n-Butanol is a good solvent for the extraction of histamine and other biogenic amines from tissues and biological fluids deproteinized by perchloric acid [1]. By washing the *n*-butanol extract with salt-saturated alkaline solution, amino acids can be removed. The specificity of the present method was examined for some biogenic amines. Polyamines, catecholamines and indoleamines did not interfere even if added to plasma in amounts more than 5 times normal.

The recovery of histamine was checked by adding known amounts of histamine (5—50 pmole/ml) to pooled plasma. Recoveries were 96 ± 4%. The precision of the method was examined by performing 20 assays on pooled plasma containing 25 and 50 pmole/ml histamine. The standard deviations were 1.34 and 2.4, respectively. The coefficients of variation were 4.9% for 50 pmole/ml and 5.6% for 25 pmole/ml histamine, respectively.

The amount of histamine in the plasma of 10 healthy men (26—29 years) determined by this method was 0.61 ± 0.16 ng/ml, which was in good agreement with 0.62 ± 0.3 ng/ml and 0.69 ± 0.26 ng/ml which were described by Graham et al. [7] and Lorenz et al. [9], respectively.

The present HSLC method for the determination of plasma histamine has the following advantages over other methods, including the enzymatic isotopic method [10]: analyses are carried out rapidly with small amounts of sample and the sensitivity can be greatly improved.

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Note

New direct micro-method for determination of valproic acid in serum by gas chromatography

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The determination of anticonvulsant serum concentration is of great importance for correct treatment of epileptic patients, in particular when it can be measured in short intervals at the beginning of or after a change in therapy. It allows the monitoring of the serum concentration, which is not always dose dependent and therefore not always predictable. In recent years, valproic acid* (dipropylacetic acid, DPA) has found increasing use in the treatment of generalized epilepsy, especially of the petit mal type in children (for a review see refs. [1] and [2]). Specific and rapid methods for routine analyses have been developed to determine DPA-serum concentrations in epileptic patients. Schmidt et al. [3] have presented a good review of the main gas chromatographic (GC) methods available. A serious drawback of all known methods, however, is the relatively large volume of serum needed, which is of special importance for newborns and infants (the repeated drawing of venous blood is difficult for technical and psychological reasons) and the rather tedious procedure, involving solvent extraction followed by evaporation.

Therefore we have searched for a new method without the above mentioned disadvantages, that is, a method which requires less serum and does not require extraction steps. The method which we describe in this paper does not require more than 20 μl of serum obtained from blood drawn from the finger trip. A 1-μl volume of this serum is applied after acidifying directly into the gas chromatograph.

MATERIALS AND METHODS

Dipropylacetic acid (sodium salt) was used in the form of the commercial 30% solution (Ergenyl®, Labaz, Düsseldorf, F.F.R.). 2-Ethyl-2-methyl-caproic acid (internal standard) was obtained from Fluka (Neu Ulm, G.F.R.). Micro

*Other nomenclature: dipropylacetic acid, 2-propyl-valeric acid, 2-propyl-pentanoic acid.

hematocrit tubes were from Dade (Miami, Fla., U.S.A.) and a micro-capillary centrifuge from IEC (Boston, Mass., U.S.A.).

Preparation of samples

Serum may be obtained from venous or from capillary blood. In the latter case the blood from the finger tip is drawn into a micro hematocrit tube (40 μl) and centrifuged in a microcapillary centrifuge. After the capillary has been broken off, the serum part is blown into a small vial. To 20 μl of serum 10 μl of 1 *N* HCl containing the internal standard 2-ethyl-2-methyl-caproic acid (0.3 mg/ml) are added. After briefly mixing, 1 μl is injected directly into the gas chromatograph. A standard working curve is prepared by adding known amounts of DPA to normal sera. A 5-μl volume from different stock solutions of DPA, made up in distilled water, is added to 15 μl of serum to obtain the different concentrations (10—150 ug/ml). The standard sera are handled in the same way as described above. The peak area ratios of the internal standard relative to DPA are plotted against the corresponding drug concentrations.

Gas chromatography

A Varian 2740 gas chromatograph was used, equipped with a glass column (6 ft. × 1/4 in. O.D.) packed with 5% DEGS-PS on Supelcoport (100—120 mesh) (Supelcoport, Bellefonte, Pa., U.S.A.). The injection temperature was 200°, detector temperature 200° and column temperature 135° isothermal. Nitrogen flow-rate was 30 ml/min, hydrogen flow-rate 40 ml/min and air flow-rate 400 ml/min. The attenuation was $(4 \cdot 10^{-11}) - (8 \cdot 10^{-11})$.

RESULTS AND DISCUSSION

A typical gas chromatogram of a serum sample is shown in Fig. 1. The retention time for DPA is 2 min and for the internal standard ca. 2.5 min. The calibration curve is linear in the range 10—150 μg/ml serum (the assumed therapeutic range is within this range [4,5]), passing through the origin. The accuracy and reproducibility of the method was evaluated by ten replicate analyses of different serum samples containing known amounts of DPA, as shown in Table I. The coefficient of variation ranged from 2.7 to 4.2%, and therefore was less than 5% which is generally accepted for quantitative analyses of antiepileptic drugs. We first compared our new method on serum samples obtained from venous blood with the method previously used in our laboratory, which was based upon a modification of common extraction procedures [6,7]. The DPA-values from both methods correspond very closely to one another. We then compared the DPA concentrations in serum obtained from venous blood with serum from blood from the finger tip (both samples from the same patient at the same time).

We did not find any significant difference between the DPA-concentrations in serum obtained from venous blood and the blood drawn from the finger tip.

The serum proteins do not interfere but become denaturated by the high injection temperature and remain in the column. Repeated use of the column does not change the retention times and only after about 300 samples does the first ca. 10 cm of the column have to be renewed. We used a normal microliter

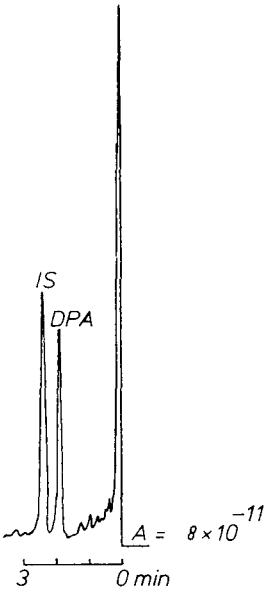

Fig. 1. Gas chromatogram of a serum sample containing DPA (76 μg/ml). Internal standard (IS) is 2-ethyl-2-methylcaproic acid; A = attenuation.

TABLE I

ACCURACY AND REPRODUCIBILITY OF THE GC DETERMINATION OF DPA

Sera contain different amounts of the drug (n=10 for each amount). S.D. = standard deviation; C.V. = coefficient of variance.

Added drug (μg/ml)	Mean	S.D.	C.V. (%)
25	25.4	1.1	4.2
50	51.7	2.0	3.8
75	76.8	2.5	3.2
100	98.9	2.6	2.7

syringe (Hamilton, 701), which is rinsed with distilled water after each sample to prevent plugging of the metal tip by the denaturated serum proteins. Normal serum constituents do not interfere, and the short chain fatty acids especially (which may accumulate as a result of an inborn error in amino acid metabolism) are eluted before DPA appears. Also there is no interference from other anti-consulvants such as phenytoin, carbamazepine, primidone, phenobarbital and ethosuximide, which we tested on patients with multitherapy.

The amount of serum required is actually not more than that which is needed for injection. To prevent too much dilution when the sample is acidified and the internal standard is added, and to retain good accuracy and reproducibility, we used 20 μl of serum.

Our method is rapid and easily practicable and is suitable for clinical routine analyses.

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Note

Rapid, sensitive gas chromatographic analysis of 8-methoxypsoralen in human plasma

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8-Methoxypsoralen (8-MOP) is increasingly used in the treatment of psoriasis. Quantitative analysis of 8-MOP in biological fluids has been performed by measurement of the radioactivity of the drug in combination with thin-layer chromatography (TLC) [1]. A spectrophotometric method employing 5 ml of plasma has been described [2]. A sensitive, simple high-performance liquid chromatographic (HPLC) technique using 4 ml of plasma [3] and gas—liquid chromatographic (GLC) methods [4, 5] with a flame-ionization detector (FID), including TLC clean-up of extracts from 2—10 ml plasma have been described in the literature for quantifying 8-MOP levels. Unfortunately these methods are time-consuming. Recently a determination of 8-MOP by electron-capture gas chromatography was described [6]. However, this method requires plasma volumes of 2 ml and a lengthy extraction procedure. The aim of this study therefore was to develop a very rapid, simple method for determining 8-MOP in small plasma samples of 0.1—0.5 ml.

EXPERIMENTAL

Reagents and chemicals

8-MOP and the internal standard 5,8-dimethoxypsoralen (Fig. 1) were of analytical grade. ^{14}C-labelled 8-MOP (specific radioactivity 2 μCi/mg) was synthesized in the isotope laboratory of the Biochemical Department of the Dr. Karl Thomae GmbH. It was labelled at the methoxy-position. The toluene used was from Mallinckrodt (Wesel, G.F.R.), nanograde quality, No. 8092; the hydrochloric acid was purchased from Merck (Darmstadt, G.F.R.), p.a. quality, No. 319.

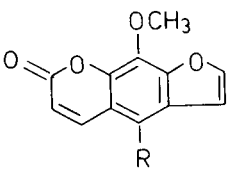

Fig. 1. Structural formulae of furocoumarins: 8-MOP = 8-methoxypsoralen; internal standard, IS = 5,8-dimethoxypsoralen.

Apparatus

The gas chromatograph used was a Perkin-Elmer F 22 equipped with a ^{63}Ni electron-capture detector (ECD) with pulse-frequency modulation. A glass column (2 m × 2 mm I.D.) was used, filled with Chromsorb 750, (80—100 mesh; Pierce, Rockford, Ill., U.S.A.), coated with 3% OV-17 (Perkin-Elmer, Überlingen, G.F.R.). The column was conditioned at 280° for three days, silanization was effected with Silyl-8 (Pierce). Operating conditions were: column-temperature, 220°; injector-temperature, 230°; detector-temperature, 250°; carrier gas, argon—methane (95:5). The integrator was a Hewlett-Packard 3380 S.

Analytical procedure

The blood was sampled via Braunülen® (Braun, Melsungen, G.F.R.) heparinized and centrifuged in glass tubes. The plasma was pipetted-off and stored frozen. It was thawed at room temperature, and 0.5-ml portions were pipetted into 10-ml glass-stoppered test-tubes, containing 0.5 ml of 0.2 *N* HCl and 2 ml toluene (containing 200 ng/ml internal standard), and mixed 15 min on a shaking-machine. After centrifuging, the plasma-phase was frozen at - 20°, the organic phase transferred into another tube and evaporated to about 200 µl in a thermoblock at 40° under a gentle stream of nitrogen. If the tubes were brought to dryness, the residue was reconstituted in 200 µl of toluene. From each of those samples 4 µl were injected into the gas chromatograph. The calibration curve was constructed by adding 50—500 ng 8-MOP (dissolved in 0.1 ml methanol) to plasma.

RESULTS AND DISCUSSION

The use of an ECD [6, 7] instead of an FID [4], produces an increase in the sensitivity and selectivity. This means that the volume of the sample can be reduced from 0.5 ml to 50 µl if suitably small tubes are used.

To avoid interfering peaks in the chromatograms, Ehrson et al. [6] used a clean-up procedure which takes advantage of the ring opening of 8-MOP at the lactone position. This procedure is time consuming. If the extraction is performed from an acidic medium, the blanks are much lower, as is shown in Fig. 2. These blanks correspond to a concentration of only 5—10 ng/ml. We analysed about 30 different human plasma samples and found this interference to be constant for these individuals. We have strong evidence, that there is no ring-opening of 8-MOP in plasma which could be re-lactonised during our acidic extraction procedure:

Firstly, plasma from a patient who had received 40 mg 8-MOP, was extracted

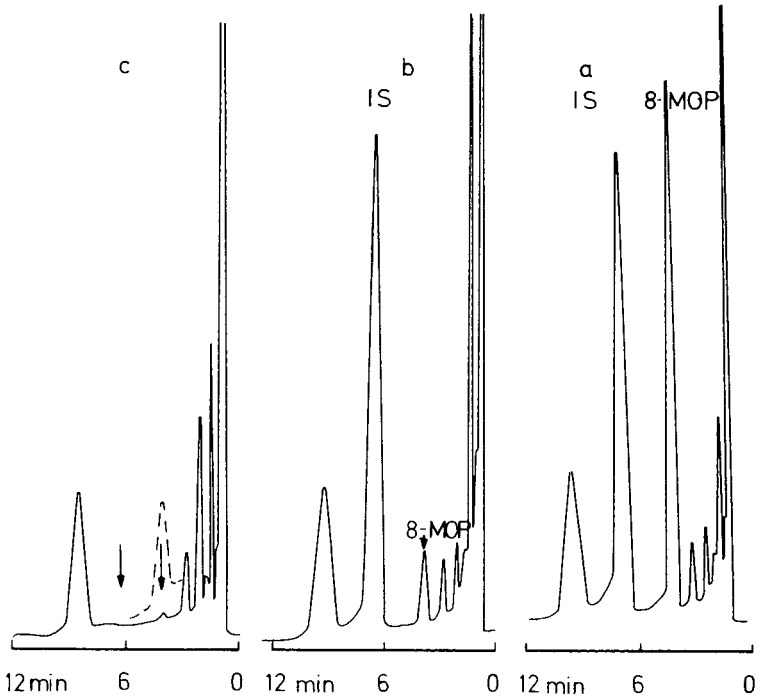

Fig. 2. Chromatograms of 8-MOP showing human plasma at a level of: (a) 500 ng/ml; (b) 50 ng/ml; (c) blank. The dotted line in (c) shows the blank for extraction at neutral pH.

at a neutral pH with toluene to prevent lactonisation and then the organic phase was washed with hydrochloric acid. With this method the plasma level was the same as that measured after our direct extraction method.

Secondly, during our metabolic studies [1] we were able to demonstrate (by structure elucidation of urine metabolites) that there is only an insignificant ring cleavage at the lactone-position of 8-MOP in man.

Thirdly, Ehrson [6] could not detect this open lactone molecule by ion-pair extraction, even at low levels.

By liquid scintillation counting, the recovery of ^{14}C-labelled 8-MOP was 98.6 ± 0.9% for n = 5 at a concentration of about 900 ng/ml. With GLC (using the method described for the construction of the calibration curve) the recovery was at a level of 100 ng/ml 8-MOP 100 ± 2.6% for n = 5 and at a level of 500 ng/ml 8-MOP 96.9 ± 1.8%, also for n = 5.

The calibration curve is linear, between 50—500 ng/ml 8-MOP. This range is attained with therapeutic doses. The reproducibility over a period of one day was studied in a concentration range of 50—500 ng/ml 8-MOP. The reproducibility over several days was studied by preparing 15 plasma samples containing 500 ng/ml 8-MOP and analysing them on different days. The results of these experiments show a good reproducibility (Table I).

However, there are three points that have to be taken into consideration to obtain good results. Firstly, some types of plastic material give rise to interfering peaks in the gas chromatogram. We therefore used Braunülen for collecting the blood and centrifuged it in glass tubes. A further point of consideration

TABLE I

REPRODUCIBILITY OF 8-MOP DETERMINATION ON DAY (A) AND BETWEEN DAYS (B)

Plasma level (ng/ml)	Mean peak area ratio	No. of determinations	S.D. (%)
(A) 50	0.076	4	4.3
100	0.149	4	2.0
200	0.285	4	0.9
300	0.412	4	1.8
400	0.527	4	2.2
500	0.654	4	2.1
(B) 500	0.650	15	2.4

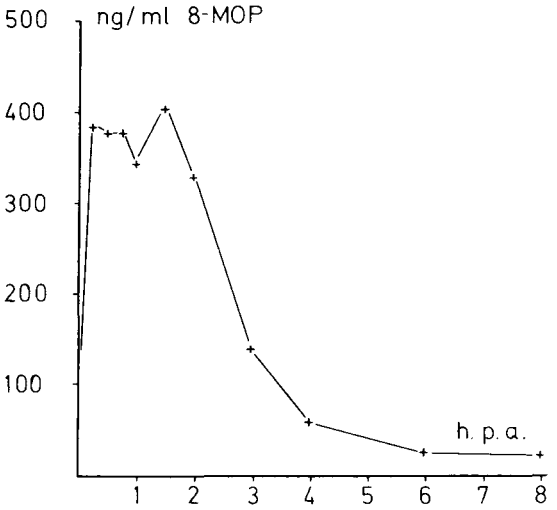

Fig. 3. Plasma concentration of 8-MOP in a human subject following p.o. administration of 40 mg 8-MOP as a solution.

is the evaporation step performed in the thermoblock. Care must be taken that the tubes are taken off the thermoblock as soon as the evaporation is complete, to prevent the observed loss of 8-MOP. Finally, a new gas chromatographic column shows some tailing. This can be reduced by injecting plasma extracts containing 8-MOP at a level of 500 ng/ml.

Fig. 3 shows the plasma concentration of 8-MOP in a human subject following p.o. administration of 40 mg 8-MOP in a solution. This plasma level is in good agreement with HPLC studies [3] and with TLC studies [1].

ACKNOWLEDGEMENT

We thank Mr. S. Weigele for his excellent technical assistance.

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Note

Gas chromatographic determination of phenazone derivatives in human plasma

I. Aminophenazone

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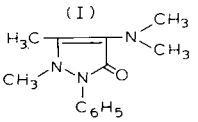
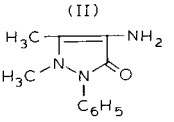
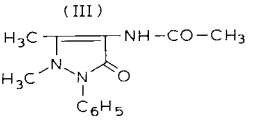
(Received March 1st, 1978)

Aminophenazone (I) is an antipyretic, analgesic and anti-inflammatory drug used in various formulations:







Several methods for the assay of I, especially by gas chromatography (GC) have been described. Windorfer and Röttger [1] reported the GC assay of I in serum, down to 1 μg/ml, with docosane as internal standard, Lavene et al. [2] described a GC method for the assay of I down to 4 μg in 5 ml plasma or 20 ml urine, and of two of its metabolites together with caffeine and barbitone. Hexobarbitone was used as internal standard. The GC of I was carried out on silicone rubber columns without derivative formulation by Fuerst et al. [3]. Vesell et al. [4] determined I in plasma and saliva down to 2 μg/ml. Aminophenazone was extracted in chloroform containing antipyrine as internal standard.

None of these techniques is sufficiently sensitive to assay I after single-dose administration. This paper describes an improved procedure which permits determinations of I down to 0.1 μg/ml.

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EXPERIMENTAL

Chemicals and reagents

Aminophenazone was supplied by Ciba-Geigy (Basle, Switzerland). 4-Aminoantipyrine was purchased from Baker (Gross Gerau, G.F.R.) and 4-acetylaminoantipyrine from Aldrich (Beerse, Belgium). Buffer pH 12 (Titrisol; Merck, Darmstadt, G.F.R.) is prepared by diluting the contents of 4 vials in 500 ml water. Chloroform, carbon disulphide and isoamyl alcohol are of analytical grade (Merck). The carbon disulphide internal standard solution contains 50 μg/ml of heneicosane (C_{21}) [Kit No. 26A; Polyscience Corporation, Niles, Ill., U.S.A.].

Equipment

The tubes to be used for the evaporation of the solvent after extraction are previously treated to prevent adsorption. They are immersed for a few seconds in a silicone (Siliclad: Clay Adams, Parsippany, N.J., U.S.A.) bath [1% (v/v) aqueous solution], rinsed first with tap water and then demineralized water, and lastly dried at 100°.

A gas-chromatograph (Fractovap 2400 T; Carlo Erba), equipped with a flame-ionization detector is used. The peak areas are recorded by an electronic integrator (Infotronics, CRS 204). The column is operated at 215°, the injector at 250° and the manifold at 270°, with a nitrogen flow-rate of 40 ml/min. Glass columns are washed with 1.0 *N* hydrochloric acid, distilled water, acetone and benzene, then silanized with a 1% (v/v) solution of hexamethyl disilazane in benzene. After this treatment, the columns are washed again with benzene and dried at 100°. The column packing is 5% SE 30 on Chromosorb W, 80—100 mesh (Applied Science Labs, State College, Pa., U.S.A.).The filled column (2 m × 3 mm I.D.) is gradually heated up to 260°. The temperature is then increased 6—8 times from 150 to 250° in about half an hour and 20 μl of Silyl 8 (Pierce, Rockford, Ill., U.S.A.) is injected during every cycle.

Extraction

A 50-μl sample of the internal standard solution (50 μg/ml) is measured into a glass tube. The solution is taken to dryness under a nitrogen stream. A 1-ml volume of the sample, 2 ml of pH 12 buffer, 1 ml of 0.1 *N* NaOH and 5 ml of a 1% solution of isoamyl alcohol in chloroform are introduced into the tube, which is stoppered and shaken mechanically (Infors) for 20 min at 200 rpm and centrifuged at 4800 *g* for 15 min. The aqueous phase is pipetted off and discarded and an aliquot of the chloroform phase is transferred to a silicone-treated tube and taken to dryness under a nitrogen stream in a dry bath at 30°.

Gas chromatography

The dry residue is dissolved with 100 μl of carbon disulphide and the tube is shaken on a mixer (Vortex).

A 2-μl portion of the carbon disulphide solution is injected into the gas chromatograph by the solvent-flush technique. It is necessary to raise the oven temperature after 10 consecutive injections to 250° for half an hour to wash out plasma residues from the column.

The content of I is calculated from the peak-area ratio by reference to a cali-

bration curve. This curve is plotted on the basis of a 0.1*N* NaOH solution containing 50 μg/ml of I. Aliquots of this solution are transferred to tubes and plasma is added to yield plasma solutions containing 0.10—5.00 μg/ml.

RESULTS AND DISCUSSION

Precision and recovery

Table I gives the results obtained when the described procedure was applied to spiked plasma samples. Concentrations down to 100 ng/ml can be accurately determined. This sensitivity is not obtained when the glass tubes are not treated with silicone.

TABLE I

PRECISION AND RECOVERY OF THE DETERMINATION OF AMINOPHENAZONE APPLIED TO SPIKED HUMAN PLASMA SAMPLES

Amount added (ng/ml)	Amount found (ng/ml)	Mean	Precision reproducibility CV (%)	Recovery accuracy
100	78			78.8
100	94			94.0
100	83			83.0
100	85	86	10.6	85.0
100	77			77.0
100	100			100.0
200	189			94.5
200	192			96.0
200	203	189	6.4	101.5
200	194			97.0
200	171			85.5
500	482			96.4
500	533			106.6
500	522			104.4
500	436	476	9.6	87.2
500	461			92.2
500	419			83.8
1000	967			96.7
1000	958			95.8
1000	970	972	3.7	97.0
1000	1029			102.9
1000	990			99.0
1000	921			92.1
5000	4837			96.7
5000	4675			93.5
5000	4789			95.8
5000	4951	4920	4.6	99.0
5000	4931			98.6
5000	5338			106.8
			Mean	94.3 ± 8.2

Plasma interference

Fig. 1 shows the chromatograms of an extract of human plasma and of the same extract spiked with 4 μg of I and 2.5 μg of internal standard. There is no interference of the normal components of the plasma extract.

Metabolites

Several metabolites of I have been described by various investigators and reviewed recently by Van Ginneken [5] and Steltzer [6]. Most have been identified by thin-layer chromatography or combined gas—liquid chromatography—mass spectrometry in urine of animals and man. 4-Amino-antipyrine (II) and 4-acetylamino-antipyrine (III) are the main unconjugated metabolites described by Lavene et al. [2], Gradnik and Fleischmann [7], Momose and Suji [8], and Oehne and Schmid [9].

Plasma spiked with these two metabolites was processed under the conditions described for the assay of I. No interference from II or III was detected. The other known metabolites of I (4-methyl-amino-antipyrine, 4-hydroxy-antipyrine, rubazonic acid and methylamino-rubazonic acid) have been found in small amounts in urine, but they have not been reported in plasma. Therefore, the proposed method can be considered specific for the unchanged drug.

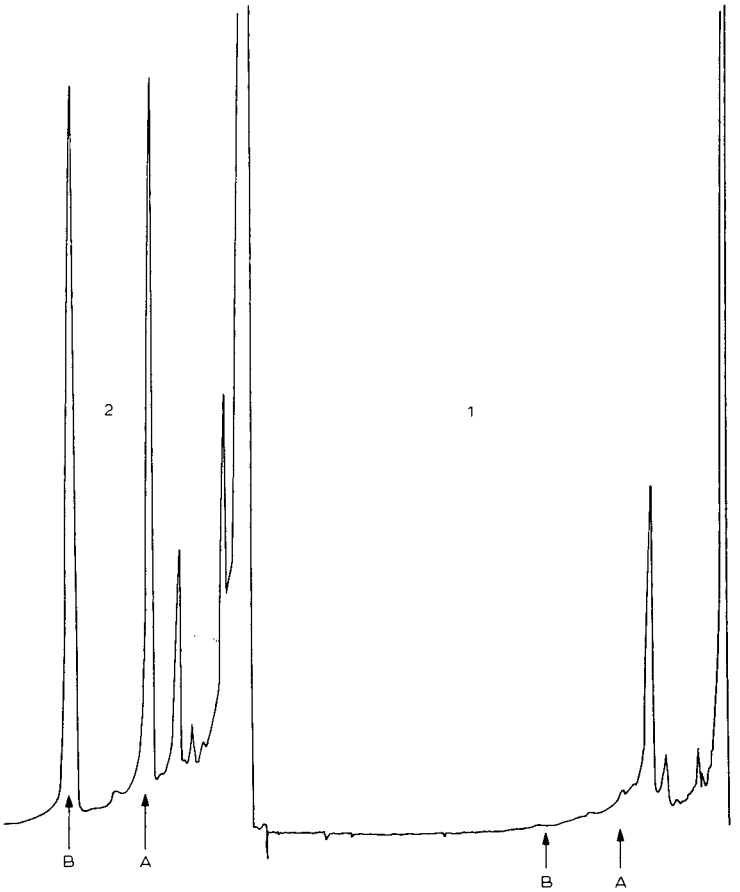

Fig. 1. Examples of chromatograms: 1 = human plasma blank; 2 = 4 μg/ml of aminophenazone (A) and 2.5 μg/ml of internal standard (B) in human plasma.

Human experiment

This method was applied to the determination of I in the plasma of one volunteer who had received one 300-mg tablet aminophenazone daily for three days. The results are shown in Table II. The plasma half-life of I was calculated from the data of the first day and found to be 2.0 h, in agreement with the value reported by Vesell et al. [4].

TABLE II

AMINOPHENAZONE PLASMA CONCENTRATIONS AFTER DAILY ADMINISTRATION OF 300 mg TO ONE VOLUNTEER FOR THREE DAYS

N.D. = not detectable.

Day of drug administration	Hours after administration	Aminophenazone plasma concentrations (μg/ml)
	0	N.D.
	2	3.3
1	4	1.3
	6	0.6
	8	0.3
	0	0.1
	2	3.9
3	4	1.1
	6	0.8

CONCLUSION

The proposed technique permits the rapid assay of aminophenazone in plasma with an adequate degree of accuracy and specificity. It is sufficiently sensitive to measure plasma levels after single-dose administration.

ACKNOWLEDGEMENTS

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Note

Gas chromatographic determination of phenazone derivatives in human plasma

II. Propyphenazone

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Propyphenazone, 4-isopropyl-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one (isopropylphenazone or 4-isopropylantipyrine), is an antipyretic, analgesic and anti-inflammatory agent. To permit the study of the bioavailability of propyphenazone, a method of measuring its concentration in plasma had to be elaborated. Several methods have been described for the assay of phenazone and aminophenazone, but few of them have been tested with propyphenazone.

Brinkmann and Hengstmann [1] determined phenazone by gas chromatography (GC) with a flame ionization detector (FID), using propyphenazone as internal standard. Lindgren et al. [2] studied the half-life of phenazone with 4-methyphenazone as internal standard, using FID—GC; Windorfer and Röttger [3] reported the GC determination of aminophenazone with docosane as internal standard, using temperature programming.

This communication describes the determination of propyphenazone in plasma by FID—GC, using hexacosane (C_{26}) as internal standard.

EXPERIMENTAL

Chemicals and reagents

Propyphenazone was supplied by Ciba-Geigy (Basle, Switzerland) and hexacosane (Kit No. 26A) was purchased from PolyScience Corporation, Niles, Ill., U.S.A. Buffer pH 12 (Merk 9882; Merck, Darmstadt, G.F.R.) is prepared by diluting the contents of 4 vials with water to a volume of 500 ml. The solvents used were all of analytical grade: chloroform (Merck 2447), isoamyl alcohol (Merck 979) and carbon disulphide (Merck 2214). 1% isoamyl alcohol is added to chloroform to make the extraction solvent.

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Equipment

GC assays are carried out on a Carlo Erba Fractovap 2400 T FID-gas chromatograph. The peak areas are given by an electronic integrator (Infotronics CRS 204). The glass column is washed with 1.0 *N* hydrochloric acid, water, acetone and benzene, silanized with a 1% solution of hexamethyldisilazane in benzene, then washed again with benzene and dried at 100°. The column (1 m × 3 mm I.D.) is packed with 3% Poly 1.110 (08264; Applied Science Labs., State College, Pa., U.S.A.) on Chromosorb W HP, 80—100 mesh. The filled column is gradually heated to 240°. The temperature is then increased 6—8 times from 140 to 240° in about half an hour and 20 μl of Silyl 8 (Pierce, Rockford, Ill., U.S.A.) is injected during every cycle. The column is operated at 220°, the injector at 230° and the manifold at 240°. The nitrogen flow is 40 ml/min. Under these conditions, propyphenazone and hexacosane have retention times of 4 and 13 min, respectively.

Standard solutions

The standard solution of propyphenazone is prepared by dissolving 5 mg of propyphenazone in 100 ml water (a few drops of methanol are necessary at the beginning to obtain a good dissolution). The standard solution of hexacosane (C_{26}) is prepared by dissolving 10 mg of hexacosane in 100 ml carbon disulphide. The dilutions required for these two solutions are prepared with the corresponding solvents.

Extraction

A 500-μl volume, corresponding to 2.5 μg of internal standard, is introduced into a stoppered glass tube and taken to dryness under a nitrogen stream. A 1-ml sample of the plasma to be analysed, 2 ml buffer (pH 12) and 5 ml chloroform—1% isoamyl alcohol are added. The tubes are shaken for 10 min at 250 rpm and centrifuged for 10 min at 4800 *g*. The aqueous phase is pipetted off and discarded. An aliquot of the organic phase is transferred to another tube and taken to dryness under a nitrogen stream in a dry bath at 70°. Evaporation must be effected very carefully and stopped just as the tubes reach dryness.

Gas chromatography

A 100-μl volume of carbon disulphide is added to the dry residue and the tube is shaken on a mixer (Vortex). A 2-μl portion of the resultant solution is injected into the gas chromatograph by the solvent-flush technique. It is necessary to raise the oven temperature after 10 consecutive injections up to 240° for half an hour to wash out plasma residues from the column.

The concentration of propyphenazone in the analysed plasma is calculated from the propyphenazone—internal standard peak-areas ratio by reference to a calibration curve. This curve is obtained by extraction of plasma spiked with increasing amounts of propyphenazone (from 0.125 μg to 10 μg/ml) and a constant amount of internal standard (2.5 μg/ml plasma). The reproducibility of the curve is checked every ten days.

RESULTS AND DISCUSSION

It was found necessary to add a sample percentage of isoamyl alcohol to

TABLE I

PRECISION AND RECOVERY OF THE DETERMINATION OF PROPYPHENAZONE APPLIED TO SPIKED HUMAN PLASMA SAMPLES

Amount added (ng/ml)	Amount found (ng/ml)	Mean	Precision reproducibility CV (%)		Recovery accuracy
125	134				107.2
125	135				108.0
125	128	129	4.9		102.4
125	121				96.8
500	524				104.8
500	502				100.4
500	500	502	3.2		100.0
500	485				97.0
2000	2071				103.6
2000	2014				100.7
2000	1916	1990	2.2		95.8
2000	1973				98.7
2000	1950				97.5
2000	2010				100.5
8000	7657				95.7
8000	7550				94.4
8000	7904	7770	2.6		98.8
8000	7985				99.8
				Mean	100.1 ± 3.9

chloroform to obtain a good extraction reproducibility. This reproducibility has been tested. Table I records the precision and recovery results for this propyphenazone GC method. It shows that a good reproducibility was obtained for 0.125 μg of propyphenazone. Unfortunately, less than 0.125 μg of propyphenazone per ml of plasma cannot be determined accurately, because the peak height is too small and the chemical background limits the detection. The limit of sensitivity of the method can consequently be taken as 0.125 μg/ml plasma.

Figs. 1 and 2 show the chromatograms obtained with a blank plasma and with the same plasma containing 0.125 μg propyphenazone per ml. Since the metabolism of propyphenazone is not known, no definitive conclusions can be drawn about the specificity of the GC assay. Nevertheless, in all bioavailability studies performed according to the technique described here, no disturbing peak appeared near propyphenazone or the C_{26} internal standard. Other hydrocarbons with a shorter chain cannot be used as internal standard; docosane (C_{22}) is not well separated from propyphenazone, and tetracosane (C_{24}) has the same retention time as a compound present in the plasma of subjects receiving propyphenazone, which could be a metabolite of the drug.

APPLICATIONS

The bioavailability of propyphenazone from different formulations was studied by this method in healthy subjects. Fig. 3 shows a curve obtained from the plasma of a subject given 220 mg of propyphenazone orally. The sensitivity

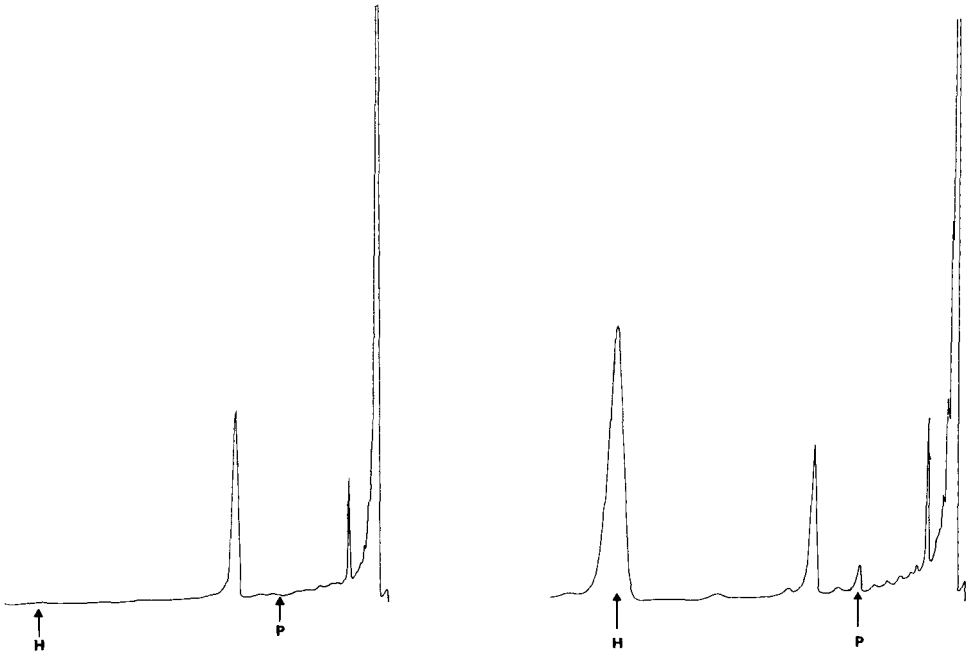

Fig. 1. Chromatogram of a plasma blank (1 ml). P = propyphenazone; H = hexacosane.

Fig. 2. Chromatogram of propyphenazone (P; 0.125 μg/ml) and hexacosane (H; 2.5 μg/ml) extracted from control plasma.

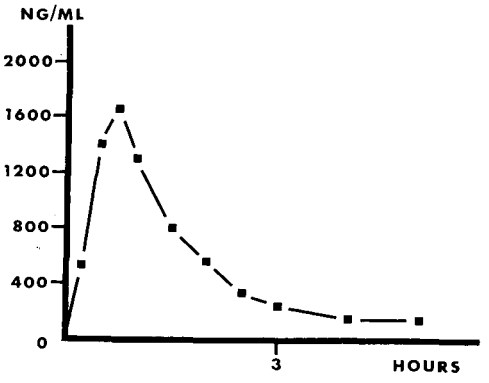

Fig. 3. Plasma concentration of propyphenazone in one subject given 220 mg of the drug orally.

of the method thus appears sufficient to determine propyphenazone in bioavailability assays.

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Note

Quantitative analysis of 5-fluorouracil in human serum by selected ion monitoring gas chromatography—mass spectrometry

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5-Fluorouracil (5-FU) is a pyrimidine analog currently used in the treatment of cancer of the breast, ovary, and gastrointestinal tract. Since its clinical introduction more than fifteen years ago, a great deal of experience has been gained in using this drug in many different dose schedules by various routes of administration, both as a single agent and as part of combined regimens, Nevertheless, a basic understanding of the clinical pharmacokinetics has been lacking, particularly with regard to the concentration—time relationship which determines adequate therapeutic effect or unacceptable toxicity. Thus, there has been a lack of pharmacological rationale for the many schedules currently used [1].

The approach to the study of 5-FU pharmacokinetics has been hampered by the fact that 5-FU has a relatively short half-life with serum levels dropping below 1μg/ml soon after a standard dose (500 mg/m^2) [2]. Until recently, the analytical methodology capable of measuring 5-FU at levels lower than 1 μg/ml has been unavailable [3]. Reported methods for the analysis of 5-FU in physiological fluids have included gas—liquid chromatography (GLC) [4—6] and more recently gas chromatography—mass spectrometry (GC—MS) [7—9]. These methods, particularly those by GC—MS, provide the sensitivity needed to

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measure 5-FU in the range found clinically. Unfortunately, the sample preparation from physiological fluids has given 5-FU recoveries of 40—75% depending on the method used. A method permitting greater recovery would be desirable.

In this report, a method which provides for quantitative recovery of 5-FU from serum is described.

MATERIALS AND METHODS

Chemicals

5-FU was obtained as a gift from Hoffman-LaRoche (Dr. E. Miller, Nuttey, N.J., U.S.A.). Phenanthrene, acetonitrile, and methylene chloride were purchased from Fischer Chemicals (Fair Lawn, N.J., U.S.A.). The acetic acid was obtained from J.T. Baker (Phillipsburgh, N.J., U.S.A.). The silylating reagent, bistrimethylsilyltrifluoroacetamide (BSTFA) were purchased from Regis (Chicago, Ill., U.S.A.).

Serum samples: standard and patient samples

Serum standards (100, 200, 300, and 400 ng/ml) were prepared in quadruplicate by adding appropriate amounts of 5-FU to pooled human serum, such that the total volume of drug plus serum was 1.0 ml. The pooled serum was obtained from healthy male adults on no medication.

Serum samples were obtained from three patients with colon cancer being treated with 500 mg/m^2 of 5-FU. Two of the patients received the drug intravenously, while the third was given 5-FU by the oral route. Serum samples were obtained prior to receiving drugs and at various time intervals following administration.

Preparation of samples for analysis

Prior to the analysis of 5-FU in serum samples, a clean-up step was required to remove proteins and related compounds which interfere with the chromatographic separation and quantitation of 5-FU. The following procedure was developed to provide a relatively clean sample for GC—MS analysis and quantitative recovery of 5-FU from serum.

Ultrafiltration. A 1.0-ml aliquot of serum was placed in a CF 25 Amicon ultrafiltration cone (Amicon, Boston, Mass., U.S.A.) and centrifuged at 2000 *g*. The contents remaining in the cone were washed twice with 1.0 ml of deionized water and recentrifuged into the same collection tube. The pH of the ultrafiltrate was adjusted to 13 with 1.0 *N* KOH.

Anion exchange resin. The alkalinized ultrafiltrate was then quantitatively transferred to a 50 × 9 mm column containing AG 1-X2 anion-exchange resin in the acetate form (resin regeneration procedure described previously [10]). The collection tube was rinsed twice with 1.0 ml of deionized water, with the wash being placed on the column after the sample was completely on the resin. The column was washed with 10 rinses of 5.0 ml of deionized water. 5-FU was then eluted with 25 ml of 0.1 *N* acetic acid and collected in a 50-ml beaker. This volume was concentrated to approximately 2 ml on a 60° hot-plate under a stream of nitrogen, and then quantitatively transferred to a 4.5 ml silylation vial (made from Corning 9826 culture tubes). The beaker was washed twice

with 1.0 ml of 0.1 *N* acetic acid, with the wash being added to the silylation vial. The entire volume was then evaporated to dryness on a 60° hot-plate under a stream of nitrogen. 1.0 ml of methylene chloride was added to the dry contents of the silylation vial and evaporated to remove azeotropically any remaining traces of water.

Silylation

Patient samples and 5-FU serum standards were silylated with 200 μl of a BSTFA—CH_3CN (1:1, v/v) mixture. The silylation vials were tightly sealed with a PTFE-lined screw-cap and heated at 150° for 3 min. An internal standard (phenanthrene) was dissolved in a CH_3CN and added to the sample with the silylation mixture.

Chromatographic and mass spectrometry conditions

A Hewlett-Packard 5981A gas chromatograph—mass spectrometer with a Hewlett-Packard 5933A data system (Hewlett-Packard, Palo Alto, Calif., U.S.A.) was used to analyze for 5-FU. A 1 m × 2 mm I.D. glass column packed with 3% Dexsil 300 on 100—120 mesh Supelcoport (Supelco, Bellefonte, Pa., U.S.A.) was used for the chromatography.

Volumes (4 μl) of the derivatized solution were injected into the GC—MS system. The initial temperature was 100° and was programmed to 250° at 16° per min. The helium carrier gas flow-rate was maintained at 22 ml/min. and the injection port temperature was 250°. The eluent from the chromatographic column was passed through an all glass jet separator at 300° and into the ion source of the MS. The MS was operated at 70 eV with a source temperature of 240° and the dodecapole at 110°.

Calculations

The level of 5-FU in serum was calculated from the relative weight response (RWR) of a standard 5-FU solution. The equations were:

$$\text{ng 5-FU per aliquot} = \frac{\text{Area 5-FU}}{\text{Area IS}} \times \frac{\text{ng IS}}{\text{RWR std}}$$

where:

$$\text{RWR std} = \frac{\text{Area 5-FU std}}{\text{Area IS}} \times \frac{\text{ng IS}}{\text{ng 5-FU std}}$$

and IS = internal standard; area = area of peak on chromatogram; ng = nanograms of compound; RWR std = relative weight response of standard 5-FU solution to an internal standard.

RESULTS AND DISCUSSION

Mass spectrum of 5-FU

In Fig. 1, the mass spectrum of the trimethylsilyl derivative of 5-FU is presented. The molecular ion is at *m/e* 274.1, and the base peak is at 259.1. From the spectrum, the *m/e* 259.1 ion was selected for quantitation of 5-FU in plasma samples. To determine the location of 5-FU on the selected ion chromato-

grams and to provide specificity to the analysis, *m/e* 273.1 and 274.1 were also monitored and the 273.1:259.1 and 274.1:259.1 ratios calculated.

A selected ion chromatogram of a standard solution containing 5-FU and 5-fluorocytosine (5-FC), a compound with similar GLC and MS characteristics,

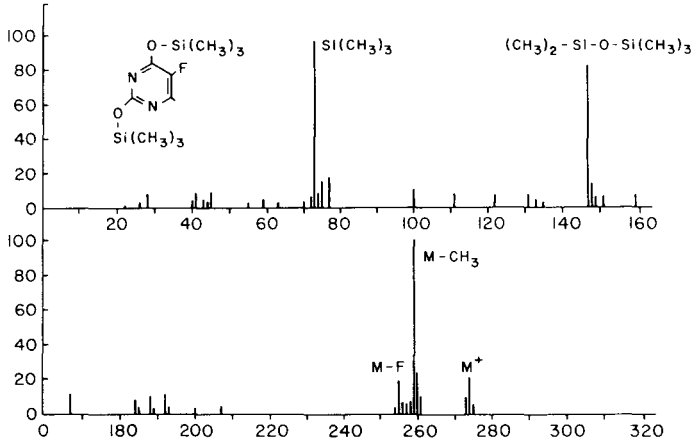

Fig. 1. Mass spectrum of 5-fluorouracil.

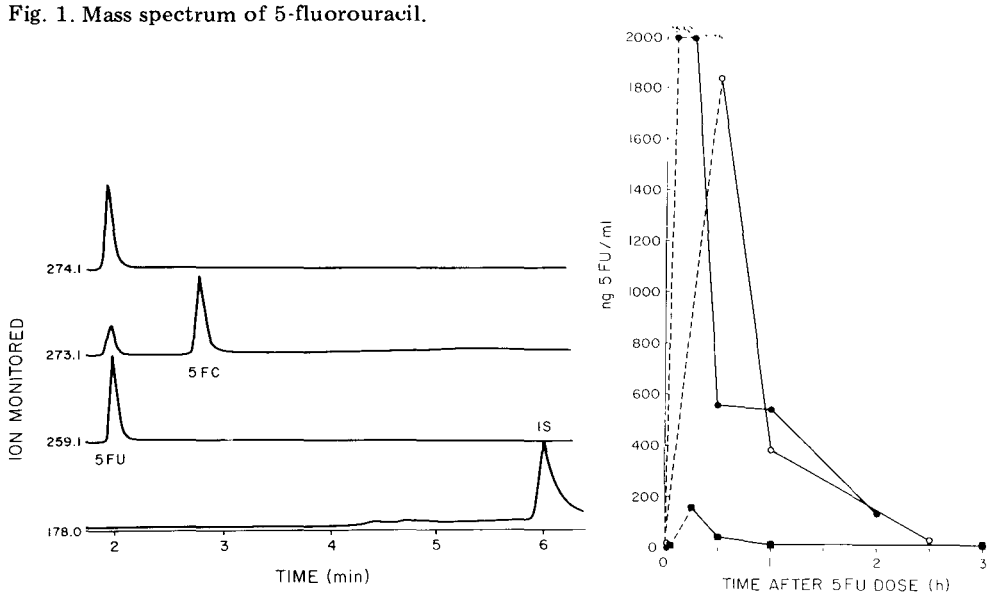

Fig. 2. Selected ion chromatograms of 5-FU, 5-FC, and phenanthrene (IS). Sample: 100 ng each 5-FU, 5-FC, and IS silylated with 200 μl CH_3CN—BSTFA (1:1, v/v) at 150° for 3 min. GLC conditions: column, 3% Dexsil 300 on 100—120 mesh Supelcoport, 1 m × 2 mm I.D., glass; initial temperature, 100°; program rate, 16°/min; final temperature, 250°; helium flow-rate, 22 ml/min; injection, 4 μl. MS conditions: separator temperature, 300°; source temperature, 240°; dodecapole temperature, 110°; ionizing voltage, 70 eV.

Fig. 3. 5-FU serum concentration in three cancer patients after administration of dose of 500 mg/m^2. Sample preparation as outlined in text. GC—MS conditions as given in Fig. 2. •, ○, 475 mg 5-FU, i.v. push; ▪, 400 mg 5-FU, p.o.

along with the internal standard, phenanthrene, is shown in Fig. 2. The highest detected voltage for each of the four selected ions (*m/e* 178.0, 259.2, 273.1 and 274.1) on the chromatogram is normalized to 100. The peaks at retention time (t_R) 2.0 min are ion fragments of 5-FU. 5-FC has an *m/e* 273.1 in its mass spectrum, and this ion is detected at a t_R of 2.8 min. Phenanthrene has a strong *m/e* 178.0 and this ion is detected at a t_R of 6.0 min. The complete separation of the ions of the two fluorinated pyrimidines shows that interference from similar compounds is unlikely. Other pyrimidines such as uracil, thymine, or cytosine do not have ion fragments corresponding to those of 5-FU and will, therefore, not interfere with 5-FU analysis by selected ion monitoring GC—MS.

Recovery of 5-FU from serum

To determine the recovery of 5-FU from serum samples, a known amount of 5-FU was added to normal serum and carried through the analytical procedure. Standards of 5-FU at various concentrations were also analyzed to determine the RWR standard value, to evaluate the linearity of the analysis method, and to calculate the 273.1:259.1 and 274.1:259.1 ion ratio values for identification of the 5-FU peak in the serum samples.

In Table I, the average RWR standard value and the average ion ratio values are presented. The standard deviation (S.D.) and the relative standard deviation (R.S.D.) are also given. The analysis of 5-FU was found to be linear from 10 ng to 1000 ng and had a minimum detectable limit of less than 1 ng.

Also in Table I, the recovery of 5-FU added to serum samples is presented. Quadruplicate analyses at 100, 200, 300 and 400 ng 5-FU were made, and the average value at each level is given. Quantitative recovery of 5-FU was found for all samples with an overall R.S.D. of 12.

The standard RWR value was found to vary slightly day-to-day; however, in-day variations were less than 5%. A new RWR std value was determined daily, and 5-FU standards were analyzed periodically during the day to evaluate the stability of the system.

TABLE I

STANDARD RWR, ION RATIOS, AND RECOVERY FROM SERUM

RWR std = average of 20 independent analyses of 100, 200, and 300 ng 5-FU standards. %R-100, 200, 300, 400 ng = percent recovery of 100, 200, 300 and 400 ng 5-FU added to 1.0 normal human serum. Four independent analyses at each concentration.

	Value	S.D.	R.S.D.
RWR std	0.705	0.064	9
273.1:259.1 ratio	9.88	0.08	0.8
274.1:259.1 ratio	19.42	0.26	1.3
%R-100 ng	103	11	10
%R-200 ng	108	11	10
%R-300 ng	93	12	13
%R-400 ng	94	12	13

Serum 5-FU levels in patients receiving 5-FU therapy

The concentration of 5-FU in serum for the three cancer patients receiving 5-FU therapy was determined using the analytical procedure. Blood samples for each of the patients were obtained at various times following the administration of the drug. Two of the patients received the drug by intravenous push, while the third received an oral dose.

The 5-FU serum levels for the three patients plotted versus time are presented in Fig. 3. The patients receiving the drug by the intravenous route had much higher serum levels than the patient taking an oral dose. The highest concentration of 5-FU was at 5 min after intravenous administration and was greater than 7000 ng/ml. The serum levels of the free drug decreased quickly, and very little drug was detected at 3 h. This method thus permits detection of 5-FU at sufficiently low levels to enable pharmacokinetic analysis.

NOTE ADDED IN PROOF

In a recent article [11], a method for the determination of 5-FU in plasma by electron-capture GLC was described. The technique has sufficient sensitivity for nanogram detection, and the sample preparation procedure gave over 80% recovery of 5-FU. No pharmacokinetic data were given.

ACKNOWLEDGEMENTS

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Note

Rapid determination of 1-(2-tetrahydrofuryl)-5-fluorouracil in human blood by high-pressure liquid chromatography

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1-(2-Tetrahydrofuryl)-5-fluorouracil (FT-207) and its major metabolite, 5-fluorouracil (5-FU), are chemotherapeutic agents frequently used in the treatment of various cancers. The analysis of 5-FU in plasma has been achieved using low-pressure column chromatography on Sephadex G-10 [1] and also by gas—liquid chromatography [2]. Fujita et al. [3] have already determined the levels of both FT-207 and 5-FU in circulating blood using a biological assay. The bioassay is probably the most commonly used technique for measuring the concentration of FT-207 in blood. Since interference by other antibiotic drugs limits the specificity of this test, we investigated the utility, sensitivity and specificity of a high-pressure liquid chromatographic (HPLC) technique for measuring FT-207 levels in human blood.

EXPERIMENTAL

All analyses were performed using a high-pressure liquid chromatograph from Waters Assoc. (Milford, Mass., U.S.A.). An M 6000A pump and a U6K injector were coupled to a μBondapak C_{18} (particle size, 8—10 μm column 300 × 4 mm I.D.). FT-207 was quantified by measuring absorbancy at 280 nm with a Waters Assoc. UV detector (Model 440) and recorded on a 10-mV full-scale National pen recorder VP-6521W. Blood concentrations of FT-207 were determined from peak-height ratios with a known amount of 6-mercaptopurine (6-MP) added as internal standard.

FT-207 was added in vitro to 0.4 ml human whole blood in amounts suitable for preparing a standard curve. After standing at room temperature for 30 min, haemolysis was completed by adding 0.5 ml water; 9 ml acetonitrile were then added to the mixtures. These were shaken vigorously for 1 min by hand and centrifuged for 5 min at 500 *g*. Seven ml of the upper phase

were transferred by pipette to another test-tube. The solvent was evaporated to dryness with a rotary evaporator at 40°. The residue was redissolved in 0.5 ml methanol and added to 0.05 ml 6-MP (0.4 mg/ml in methanol). Samples were filtered through Fluoropore filters (0.45 μm, Type FT-045, Sumitomo, Osaka, Japan), and 20-μl aliquots of the filtrate containing 0.06—1.68 μg of FT-207 were injected onto the column and eluted with 10 % aqueous methanol at a flow-rate of 2 ml/min.

For measuring FT-207 levels in blood, FT-207 was given intravenously to three cancer patients who had normal liver functions in a single dose of 16 mg per kg body weight. Blood samples were collected at 1, 3, 5, 12 and 24 h after the drug was injected. The FT-207 in 0.5 ml blood was extracted as described above and injected onto the column.

FT-207 and 6-MP were obtained from Taiho Pharmaceuticals (Tokyo, Japan) and Takeda Chemicals (Osaka, Japan) respectively. Methanol, acetonitrile and water were purchased from Wako (Osaka, Japan). All were liquid-chromatographic reagent grade.

RESULTS AND DISCUSSION

High-pressure liquid chromatograms of untreated human blood as a control and of FT-207 added to blood are shown in Fig. 1A and B. FT-207 eluted as a sharp symmetrical peak with a retention time of 7.8 min. An internal standard added to reduce the chance of pipetting errors did not interfere with the separation of this drug.

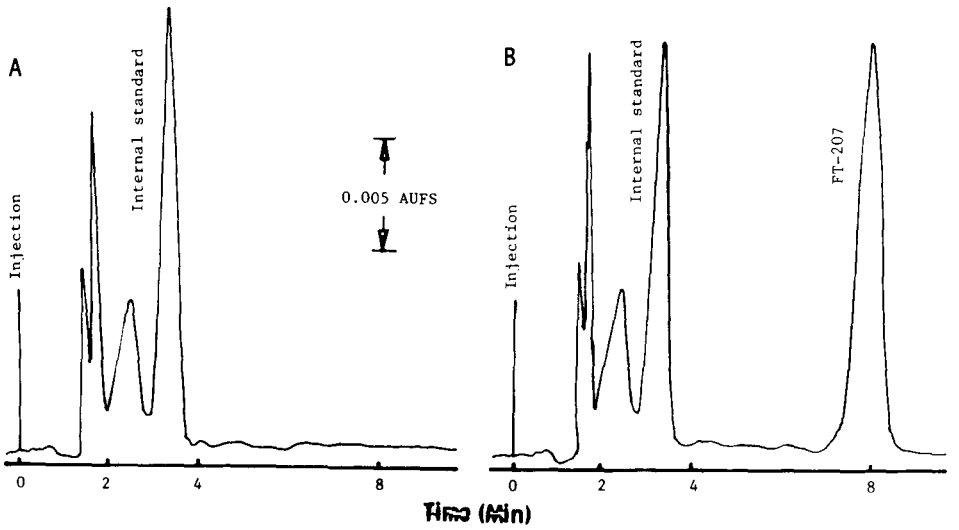

Fig.1. High-pressure liquid chromatograms of (A) human blood alone as a control, and (B) FT-207 (20 μg) added to 0.4 ml blood. Extraction procedures are described in Experimental.

The standard curve constructed from known amounts of FT-207 added to human blood is linear between at least 0.1 and 1.7 μg for FT-207. Quantitative results obtained by this procedure are given in Table I. The recovery of FT-207 extracted from whole blood and serum is approximately 100 %.

FT-207 levels in blood from three cancer patients with normal hepatic functions were determined by this technique following a single intravenous dose of FT-207 (16 mg per kg body weight). Levels were maintained at approx. 20 μg/ml up to 5 h after administration and then gradually decreased (Table II). 5-FU was similarly extracted and injected onto the column, as described for FT-207. A satisfactory recovery was obtained in the range 0.1—1.0 μg 5-FU. The method is not suitable for 5-FU, however, because 5-FU concentrations in the blood following a therapeutic dose of FT-207 or 5-FU rapidly [4] decreased below the limits of sensitivity of the method. Since it was found that blood concentrations of FT-207 at the 0.1 μg/ml level could be specifically measured in less than 15 min after a simple extraction process, this method may be useful in clinical pharmacology for detecting the efficacy and side-effects of FT-207.

TABLE I

PERCENTAGE RECOVERY OF FT-207 EXTRACTED FROM WHOLE BLOOD AND SERUM

Sample	FT-207 (μg)		Recovery (%)
	Added	Recovered (mean ± S.E.)*	
Whole blood	100	99.3 ± 2.4	99.3
	75	76.9 ± 4.1	102.5
	50	48.2 ± 1.5	96.4
Serum	50	49.9 ± 4.3	99.8

*$n = 3$.

TABLE II

BLOOD LEVELS OF FT-207 IN CANCER PATIENTS WITH NORMAL HEPATIC FUNCTIONS

Patients	FT-207 (μg/ml blood)				
	Time after administration (h)				
	1	3	5	12	24
Rectal cancer	26.8	23.0	17.4	10.5	5.6
Lung cancer	30.6	23.6	21.2	11.0	5.8
Ovarian cancer	28.6	24.2	16.3	12.7	2.5
Mean	28.7	23.6	18.3	11.4	4.6
S.E.	1.1	0.3	1.5	0.7	1.1

ACKNOWLEDGEMENTS

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Note

Comparison of high-performance liquid chromatography and a spectrophotometric technique for determining plasma warfarin

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The advent of new methods for assaying drug levels in various body fluids gives the physician a mechanism for prescribing exact dosage and thus obtaining a more desirable therapeutic:toxicity ratio. Warfarin, which interferes with the function of the four vitamin K dependent clotting factors, is often used to treat thromboembolic disorders [1]. The one-stage prothrombin time can be used to monitor the effect of this drug. However, studies have indicated that many patients will either be under- and/or overtreated with this drug, resulting in serious complications [2, 3]. Reasons for this problem include drug interactions [4, 5] age [6] and discrepancies in results from the coagulation assays used to monitor warfarin's effect [7]. An easy, accurate method of assaying plasma warfarin levels could provide better control.

This study is a portion of a Veterans Association Cooperative Study in which warfarin is used in a random fashion as adjuvant to standard neoplastic regimens. Human experimentation committees at each of the participating institutions approved the study. In determining plasma warfarin levels using the spectrophotometric assay [8] we noted sporadic results in our first group of patients. For this reason a more reliable assay was required. Many techniques were available including thin-layer chromatography (TLC) [9] fluorometry [10], gas—liquid chromatography (GLC) [11, 12] and high-performance liquid chromatography (HPLC) [13—15]. We compared HPLC and the original spectrophotometric assay, a study which has not previously been reported. In our study, HPLC was found to be extremely sensitive, easily performed, and required only small volumes of plasma. These factors led us to conclude that in the clinical evaluation of plasma warfarin levels, HPLC is the procedure of choice.

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MATERIALS AND METHODS

Citrated plasma (Vacutainer®; Becton-Dickinson, Rutherford, N.J., U.S.A.) was obtained from each individual prior to and after at least one week of warfarin therapy. The same lot number of warfarin (supplied by Endo Labs., Garden City, N.J., U.S.A.) was given to all patients. Specimens were sent frozen to the central laboratory for analysis.

The spectrophotometric assay, which requires 2.0 ml of plasma for each assay, was performed as described by O'Reilly et al. [16].

The HPLC assay of Bjornsson et al. [17] was modified as described below. An internal standard of *p*-chlorowarfarin 3-(α-acetonyl-*p*-chloro-benzyl)-4-hydroxycoumarin (Aldrich, Milwaukee, Wisc., U.S.A.) was added at a final concentration of 2.0 μg/ml to 0.5—1.0 ml of plasma. The ether extracts were evaporated in glass Concentratubes® (Laboratory Research, Los Angeles, Calif., U.S.A.) using a Myer N-Evap (Organomotion Assoc., Shrewsbury, Mass., U.S.A.) at 40°.

After the specimens were completely evaporated, 0.25 ml of methanol (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.) was used to dissolve the residues. A 20-μl aliquot was injected into the chromatographic column, a 30 cm × 4 mm I.D. μBondapak C_{18} (Waters Assoc., Milford, Mass., U.S.A.). Other equipment included a Waters Model 6000 A pump and a U6K injector. A Model 440 absorbance detector set at 313 nm with a dual channel recorder (0.02 a.u.f.s.) was used to examine the column eluate. The eluent was methanol—acetic acid—water (75:0.5:25) at a flow-rate of 1 ml/min. All solutions were filtered through a 0.50 μm Fluoropore FH 47 cm filter using a hydrosol stainless filter holder (Millipore, Bedford, Mass., U.S.A.).

RESULTS

In the HPLC system, warfarin has a retention time of 5.5 min. and that of the internal standard *p*-chlorowarfarin, 6.7 min. (Fig. 1). This relationship was constant from day-to-day, although the retention time of the individual compounds at times varied. Fig. 2 represents the results obtained when crystalline warfarin was added to pooled control plasma from 0.3 μg/ml to 5 μg/ml prior to extraction. Each point and standard deviation is based on eight separate determinations. The extraction efficiency of each compound was greater than 95%. Peak heights were determined by triangulation and plasma concentrations of warfarin by peak-height ratios.

A similar experiment using the spectrophotometric assay demonstrated that this technique was inaccurate below 0.6 μg/ml. However, the correlation coefficient (0.95) for the standard curve was similar to that observed in the HPLC assay.

Fig. 3 shows the results of the two different methods of measuring plasma warfarin. For patient 14 only the post warfarin specimen was received. Values obtained on the pre-warfarin plasmas revealed that, except for patient 6, the spectrophotometric assay demonstrated significant levels of warfarin. In patient 9, the pretreatment value was higher than that obtained on the post-warfarin treatment plasma. Patient 8 had a small amount of warfarin in his

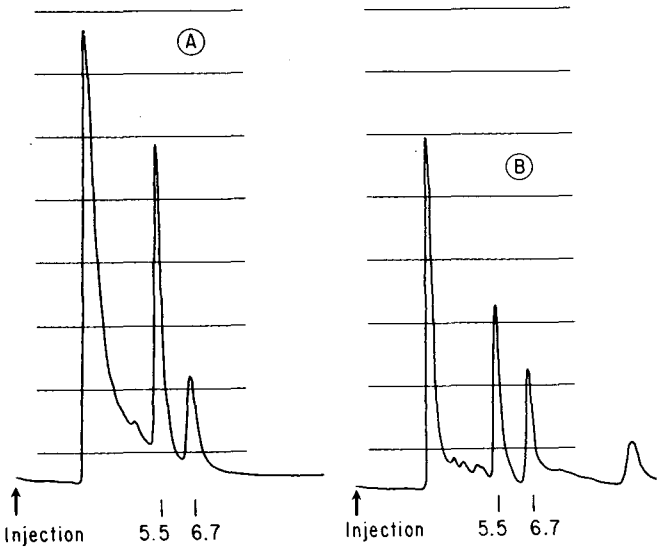
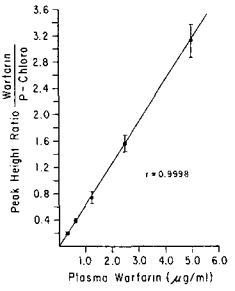


Fig. 1. HPLC of plasma extracts. (A) Normal plasma to which 1.25 μg/ml of crystalline warfarin and 2.0 μg/ml of *p*-chlorowarfarin were added prior to ether extraction. (B) A patient's plasma containing warfarin to which the internal standard has been added. Retention time for warfarin is 5.5 min. The *p*-chlorowarfarin elutes at 6.7 min. Additional peaks can be seen in the patient's sample clearly separated from warfarin.



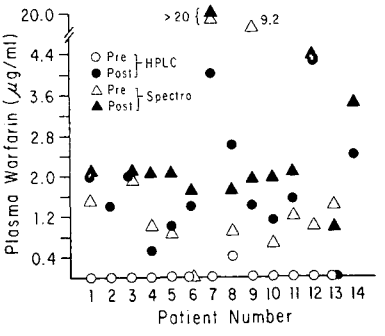


Fig. 2. Standard curve constructed by adding varying concentrations of crystalline warfarin and 2.0 μg/ml of *p*-chlorowarfarin to normal plasma prior to ether extraction and HPLC. Elution conditions are given in the text.

Fig. 3. Comparison of plasma warfarin values determined by HPLC and spectrophotometry.

pre-treatment specimen by HPLC. However, there is doubt whether plasma was actually obtained prior to beginning warfarin therapy. Patient 13 although scheduled to receive warfarin was never started on therapy. The pre- and post-HPLC values in this patient were zero, while the spectrophotometric assay suggested that this individual did have warfarin in his plasma. Only three post-warfarin specimens and one pre-warfarin specimen gave similar results by both techniques.

DISCUSSION

The use of methods which determine the presence of drugs and their metabolites in biological fluids must be accurate in the ideal as well as in the practical setting. We compared two methods for measuring plasma warfarin; HPLC and spectrophotometry. The HPLC technique was far superior. It is specific for warfarin. The chromatographic pattern for the crystalline drug and that of extracted warfarin-containing plasma were similar. The separation of the substances extracted from plasma by chromatography excludes any materials which interfere with the detection of warfarin, a process that is not part of the spectrophotometric technique. We reviewed the medical records of our patients in an attempt to determine whether any were taking medications which might account for the wide fluctuations in the spectrophotometric assay. Every individual was receiving so many drugs that it was not possible to make any correlations.

The use of the internal standard in HPLC standardizes the extraction between specimens, which cannot be done with the spectrophotometric assay. The spectrophotometric assay is inaccurate below 0.6 μg/ml, while HPLC can easily detect 0.3 μg/ml of warfarin in plasma. Another advantage of the HPLC method is that both the metabolites of warfarin, and warfarin can be detected [18].

The biological effect of warfarin, which is to interfere with the proper synthesis of the vitamin K dependent coagulation factors, is easily assessed using the one-stage prothrombin time. In the clinical setting only about one half of those on this drug achieve the appropriate level of anticoagulation. O'Reilly and Aggeler [19] in an extensive review cite many factors contributing to this problem. However, to date, correlation of plasma warfarin level and prothrombin time has not been achieved. Using the HPLC method described it is possible to examine the relationship between the biologic effect of warfarin and its concentration in plasma without interference by other drugs. It is anticipated that the HPLC method will not only assist in evaluating patients who have abnormal responses to warfarin, but in addition, the pharmacology of this compound and its metabolites can be more specifically investigated.

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NOTE ADDED IN PROOF

Methyl cyanide—acetic acid/water [46:54 (1.5%, pH 5.0)] can be used, as the eluent. In this situation warfarin has a retention time of 4.5 min and the internal standard 5.8 min.

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Note

In vitro and in vivo studies with sodium pertechnetate and technetium-labelled methylene diphosphonate

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(First received February 15th, 1978; revised manuscript received May 24th, 1978)

The isotope technetium-99m is a conveniently available generator-produced radionuclide which, together with the introduction of new chelating agents has become widely used for labelling a variety of compounds. It has favourable nuclear properties for diagnostic imaging [1]. These include a convenient γ-ray energy of 140 keV, absence of β-decay, and a short half-life of 6 h. It is eluted as sodium pertechnetate ($Na^{99m}TcO_4$) from its parent with saline from a generator consisting of ^{99}Mo adsorbed on to an alumina column.

The pertechnetate ion ($^{99m}TcO_4^-$) is considered to be the most stable chemical form [2], and it does not readily bind to chelating agents such as ethane-1-hydroxy-1,1-diphosphonate (EHPD) and methylene diphosphonate (MDP). In order to prepare a bone imaging agent, a less stable reduced state of ^{99m}Tc is usually desirable. A number of reducing substances are available, and some of these include ferrous ion [3], ferric chloride and ascorbic acid [4], sodium borohydride [5], stannous ion [6] and concentrated hydrochloric acid [7]. Stannous ion is the most popular, and it is considered to be the most suitable reducing agent for bone seeking compounds. It causes a reduction of technetium from Tc(VII) to Tc(IV) oxidation state [8].

Apart from the diphosphonates, other ^{99m}Tc-labelled radiopharmaceuticals can similarly be prepared by the stannous reduction of pertechnetate. Some of these include diethylenetriaminepentacetic acid [9], gluconate [10] and dimethylglyoxime [11]. It should be pointed out that the chemical forms of many of these labelled complexes are still unknown. In the case of dimethylglyoxime Deutsch et al. [11] have shown, using X-ray analysis, that reduction of $^{99m}TcO_4^-$ by tin(II) in the presence of this agent yields a ^{99m}Tc-Sn-di-

methylglyoxime complex in which the tin and technetium are intimately connected by a triple bridging arrangement.

Reduced technetium can be reoxidised to pertechnetate by presence of oxygen:

$$TcO_2 + 2H_2O \rightarrow TcO_4^- + 4H^+ + 3e$$

The stability of the labelled complex (^{99m}Tc—MDP) is also affected by presence of oxygen, so it is important that sufficient stannous ions are added to ensure complete reduction of the technetium. Free $^{99m}TcO_4^-$ has occasionally been detected in our ^{99m}Tc—MDP preparation in such quantities which were sufficient to produce localisation in non-osseous structures, thus decreasing the quality of the bone scan.

These experiments were carried out (i) to determine the efficiency of labelling stannous methylene diphosphonate (Sn-MDP) with free $^{99m}TcO_4^-$ by radiochromatography (ii) to demonstrate the presence of both the labelled complex and any unreduced $^{99m}TcO_4^-$ by autoradiography (iii) to compare the whole body distribution of $^{99m}TcO_4^-$ and ^{99m}Tc-labelled MDP by nuclear imaging with a γ camera and (iv) to quantitate the distribution of each agent in bone and various types of soft tissues.

EXPERIMENTAL

The preparation of Sn-MDP in the form of a freeze-dried kit and its use for labelling red blood cells have already been described [12, 13]. Each kit contains 5 mg MDP + 0.3 mg SnF_2. For labelling purposes 5 ml of eluted, pyrogen-free $^{99m}TcO_4^-$ solution containing the required amount of radioactivity were added to the stannous diphosphonate mixture in the kit vial. The mixture was shaken vigorously for 2 min to ensure complete and uniform labelling, and the solution filtered through a 0.22 μm membrane filter before use.

The radiochemical purity, i.e. the fraction of the total activity present in the stated chemical form, was determined by thin-layer chromatography (TLC) using 85% methanol as the solvent system. Pre-coated silica gel (0.25 mm thick) plastic sheets (Camlab., Hornchurch, Great Britain) were used as adsorbent. A 5-μl volume of the labelled preparation containing 5 μg MDP was spotted at a distance of 1 cm from one end of each TLC strip. This end was placed lowermost in a chromatographic tank containing a depth of 0.5 cm of the solvent. The strips were allowed to develop until the solvent front migrated to a distance of 1 cm from the upper end. The process was repeated for $^{99m}TcO_4^-$. At the end of the development the strips were dried in air. Some were cut into 5-mm sections for counting in a well-type crystal scintillation counter to determine their radioactivity content. The remainder were held in contact with Industrial "C" Kodak film by surgical tape, and the films left to expose in a dark box for 3 h. They were then developed in Kodak D. 19 developer, fixed in Kodak FX 40 fixer, and dried in an oven at 40°.

The "in vivo" distribution of $^{99m}TcO_4^-$ and ^{99m}Tc—MDP was tested in New Zealand albino rabbits weighing 2.3—2.9 kg. Each animal was injected with a dose of 150—200 μCi ^{99m}Tc activity in a marginal ear vin, and 2 h later it was placed under a Toshiba γ-camera and an image of the isotope

distribution recorded for both $^{99m}TcO_4^-$ and ^{99m}Tc—MDP. This period of 2 h from injection was chosen as it corresponds to the interval when most bone scans are performed in patients undergoing a skeletal survey.

Tissue distribution studies were carried out in Wistar rats, 150—200 g in weight. Each rat was anaesthetised with pentobarbitone sodium (30 mg/kg) intraperitoneally, and a dose (40—60 μCi) of $^{99m}TcO_4^-$ and ^{99m}Tc—MDP was injected into a tail vein in separate rats. The animals were killed 2 h later, and samples from the following organs were removed for counting with a NaI(T1) counter: bone, blood, liver, kidney, muscle, thyroid and spleen.

RESULTS AND DISCUSSION

Radiochromatograms and the corresponding autoradiographs are shown for $^{99m}TcO_4^-$ and ^{99m}Tc—MDP in Fig. 1. In Fig. 1A the peak activity occurs at some distance from the point of application of the sample on the TLC strip, and this suggests that $^{99m}TcO_4^-$ moves with the solvent front with an R_F value which was found to be 0.86. In Fig. 1B the peak appears at the origin, and this indicates that the ^{99m}Tc—MDP complex remains fixed and does not move with the solvent front during development.

The radiochemical purity of the preparation gives a measure of the yield of the product formed after labelling, and this can be referred to as the labelling efficiency where

$$\text{labelling yield} = \frac{\text{peak activity}}{\text{total activity}} \times 100\%$$

The percentage labelling yield of the ^{99m}Tc—MDP complex was found to be 96.3 ± 2.1 (mean ± S.D.; n = 10). A high labelling yield is usually desirable as this would lead to an increase in the target/non-target ratio.

The nature of the labelled complex which remains at the origin on the TLC

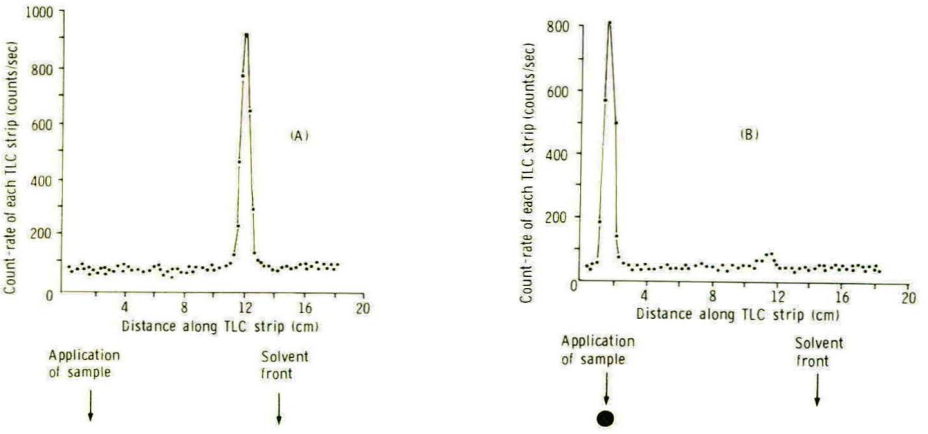

Fig. 1. Radiochromatogram and autoradiographs of (A) pertechnetate, $^{99m}TcO_4^-$, and (B) technetium-labelled methylene diphosphonate, ^{99m}Tc—MDP.

strip is not fully understood. It has been suggested that this complex might consist of some unbound or reduced hydrolysed ^{99m}Tc [14, 15]. Billinghurst [16] has suggested a method for separating free $^{99m}TcO_4^-$ and reduced hydrolysed ^{99m}Tc using a combination of acetone and saline as solvents.

Several analytical techniques are available which can be used for assessment of radiochemical impurities in technetium-labelled compounds. Among these, paper chromatography (PC) and TLC are considered to be relatively simple to perform, and they provide an estimate of the free $^{99m}TcO_4^-$ yield. For a more detailed quantitative measurement of radiochemical impurities, PC and TLC may be combined with paper electrophoresis and gel filtration. The latter techniques are not suitable for a day-to-day quality control program because of their complexity, and due to the fact that they are too time-consuming to perform.

PC was not considered in this work because of the slow development of this system which results in oxidation and subsequent streaking of the radiopharmaceutical on the paper strip. The amount of streaking on TLC silica gel is less than that on the paper as would be expected from the shorter development time.

Images recorded with the γ-camera are shown in Fig. 2. It can be seen that free $^{99m}TcO_4^-$ distributes generally in soft tissues (Fig. 2A) whereas ^{99m}Tc—MDP localises predominantly in the skeleton (Fig. 2B). In the case of the latter agent it is important that complete reduction of technetium occurs as any which remains in the unreduced state will simply be taken up by soft tissues such as the thyroid, stomach and salivary glands. This reduction is brought about by adding the optimum amount of stannous ions, as excess of reductant

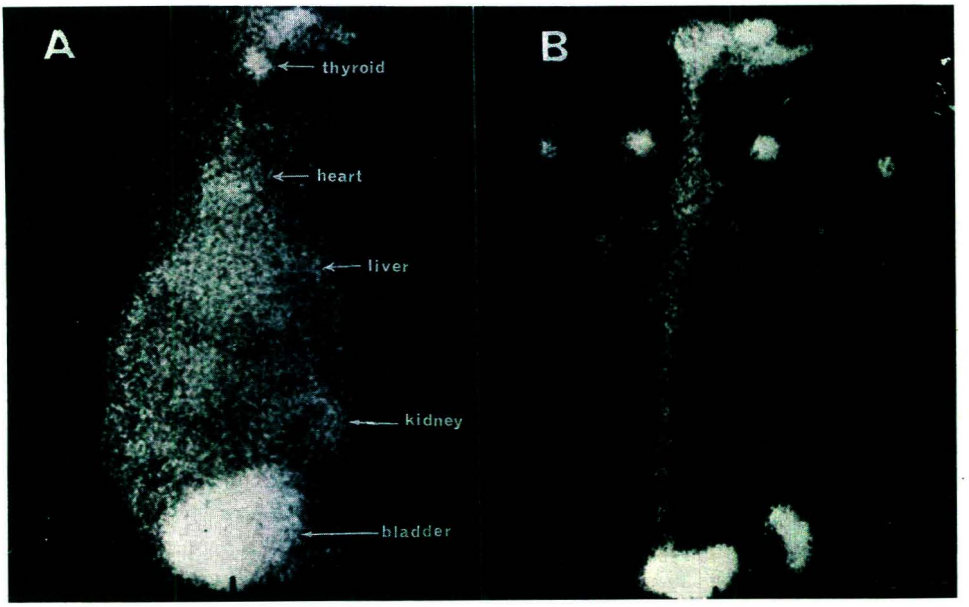

Fig. 2. γ-camera images of the rabbit taken 2 h after injection of a dose of (A) pertechnetate and (B) technetium-labelled methylene diphosphonate.

can lead to formation of technetium and stannous colloids which would then be taken up selectively by the liver and spleen:

$$2TcO_4^- + 3SnF_2 + 2H_2O \rightarrow 2TcO_2 \downarrow + 3SnO_2 \downarrow + 4HF + F_2$$

The results of the tissue distribution study are shown in Table I. In the case of free $^{99m}TcO_4^-$ a high concentration occurs in soft tissues and there is comparatively less uptake in bone. On the other hand the ^{99m}Tc—MDP combination gave the highest concentration in bone with rapid clearance from the non-osseous compartments. This is in agreement with the high labelling yield of the bone complex (96.3 ± 2.1%) as shown by radiochromatography, and with the γ-camera images shown in Fig. 2.

TABLE I

TISSUE DISTRIBUTION OF $^{99m}TcO_4^-$ AND ^{99m}Tc—MDP IN RATS WHICH HAVE BEEN KILLED 2 h AFTER INJECTION

Tissue	Percentage of dose per gram of tissue (mean ± S.D.; n = 5)	
	$^{99m}TcO_4^-$	^{99m}Tc—MDP
Bone	0.58 ± 0.12	2.54 ± 0.81
Blood	0.81 ± 0.23	0.03 ± 0.01
Liver	1.35 ± 0.44	0.04 ± 0.01
Kidney	1.29 ± 0.37	0.83 ± 0.26
Thyroid	2.71 ± 0.92	0.02 ± 0.01
Muscle	0.19 ± 0.06	0.01 ± 0.005
Spleen	0.84 ± 0.25	0.03 ± 0.01

TLC is widely accepted as a reliable "in vitro" method of testing for presence of radiochemical impurities in bone seeking radiopharmaceuticals. It is simple, rapid, inexpensive and provides complete separation of the ^{99m}Tc—MDP complex from any unreduced free $^{99m}TcO_4^-$. These impurities are likely to degrade the quality of the bone scan, increase the absorbed radiation dose to the patient, or localise in non-target areas, thus giving incomplete or misleading information to the user.

It might be argued that in vitro testing of a radiopharmaceutical is probably a poor reflection of its biological distribution. For this reason in vitro assay by radiochromatography should occasionally be checked by in vivo scintigraphy or by quantitative assessment in small laboratory animals. The combination of these techniques has been shown to yield sufficient data for routine quality control and provides useful information about the radiochemical purity of the technetium-labelled compound.

ACKNOWLEDGEMENTS

I wish to thank Dr J.P. Lavender for his help, encouragement and permission to carry out this work, Dr N.M. Spyrou for constructive criticisms and very valuable discussions and Tara Khan for typing the manuscript. This work was supported by the Cancer Research Campaign.

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Journal of Chromatography, 146 (1978) 533—537
Biomedical Applications

CHROMBIO. 203

Note

Dünnschichtchromatographische Bestimmung von Acylaminoäthyl-arylsulfonylharnstoffen im Serum nach Hydrolyse und Dansylierung

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(Eingegangen am 24. Februar 1978; geänderte Fassung eingegangen am 10. Mai 1978)

Zu den wirksamsten oralen Antidiabetika gehören die Acylaminoäthyl-Derivate von Arylsulfonylharnstoffen wie Glipizid (Glibenese®), Glibenclamid (Euglucon®), Glisoxepid (Pro-Diaban®) und Glurenorm®. Wegen ihrer niedrigen Dosis mit maximalen Serumkonzentrationen von etwa 500 ng/ml pro Tablette ist eine direkte dünnschichtchromatographische (DC) Bestimmung durch Messung der UV-Absorption bzw. der Fluoreszenzlöschung von Leuchtstoffschichten wie beim Chlorpropamid [1] nicht mehr möglich. Eine gaschromatographische (GC) Bestimmung mit Elektroneneinfangdetektion der Methyltrifluoracetyl- oder Methylheptafluorbutyryl-Derivate wie beim Chlorpropamid und Tolbutamid [2] würde wegen des grösseren Molekulargewichts und einer zusätzlichen polaren Gruppe der Acylaminoäthyl-Derivate höhere Säulentemperaturen erfordern, sodass sich Probleme hinsichtlich der thermischen Stabilität ergeben könnten. Als weitere, geeignete Methoden bieten sich an: Die Pyrolyse—GC (Tolbutamid [3], Tolazamid [4]), die Massenspektrometrie unter Verwendung eines stabilen Isotops als innerem Standard (Tolbutamid und seine Metabolite [5]) und schliesslich die radioimmunologische Methode (Glisoxepid [6]).

Die vorliegende Methode, die sich durch geringen apparativen Aufwand auszeichnet, geht davon aus, dass sich die Acylreste der Acylaminoäthyl-sulfonylharnstoffe selektiv durch alkalische Hydrolyse abspalten lassen, ohne dass dabei die Sulfonylharnstoffgruppe angegriffen wird. Nach Neutralisation der Lösung wird das freigesetzte Amin entsprechend der bewährten Methode zur Bestimmung biogener Amine aus biologischem Material [7—12] in ein fluoreszierendes Dansylderivat übergeführt und nach zwei-dimensionalen

DC-Trennung auf Kieselgel im Nanogrammbereich mittels eines Chromatogramm-Spektralphotometers ausgewertet.

Auch eine saure Hydrolyse kann durchgeführt werden, bei welcher gleichzeitig die Acylamino- und die Harnstoffgruppe gespalten werden. Da wegen der grösseren Beständigkeit der Harnstoffgruppe längere Reaktionszeiten erforderlich sind, wird die alkalische Spaltung bevorzugt. Ein Nachteil der sauren Hydrolyse ist auch, dass ev. vorhandene aliphatische Hydroxylgruppen, z.B. bei den Metaboliten, unter Bildung isomerer Olefine eliminiert werden.

Die Verhältnisse der alkalischen und sauren Spaltung werden in Fig. 1 am Beispiel von Glipizid dargestellt. Bei der sauren Spaltung entsteht als Zwischenprodukt N-(4-Aminoäthylbenzolsulfonyl)-N′-cyclohexyl-harnstoff, als Endprodukte Cyclohexylamin und 4-Aminoäthylbenzolsulfonamid. Bei der alkalischen Spaltung tritt nur der N-(4-Aminoäthylbenzolsulfonyl)-N′-cyclohexyl-harnstoff auf.

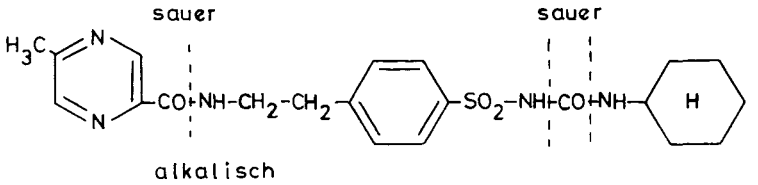

Fig. 1. Spaltung von Glipizid durch saure und alkalische Hydrolyse.

MATERIAL

Geräte

Zeiss-Spektralphotometer PMQ II, Camag-Z-Scanner, Goerz-Schreiber RE 541.

Lösungen

1 *M* Acetatpuffer von pH 4.3, hergestellt aus 1 *M* NaAc und Essigsäure; 1 *M* Carbonat—Bicarbonatpuffer von pH 9.5, hergestellt aus 1 *M* $NaHCO_3$ und 5 *N* NaOH; Dansylchloridlösung: 100 mg Dansylchlorid (Merck, Darmstadt, B.R.D.) in 50 ml Aceton.

Glipizid-Standard. 5 mg Glipizid (Pfizer Austria, Wien, Österreich) werden in 100 ml Äthanol gelöst. 1 ml dieser Lösung wird zur Trockene abgedampft. Der Abdampfrückstand wird nach (b_1) und (c) des methodischen Teils hydrolisiert und dansyliert. Der Trockenrückstand des Dansylderivates wird in 1 ml Äthanol gelöst.

Mafenid-Standard. 5 mg Mafenid (4-Aminomethylbenzolsulfonamid, Bayer-Leverkusen) werden in 100 ml Äthanol gelöst. 1 ml dieser Lösung wird zur Trockene abgedampft und der Rückstand nach (c) des methodischen Teils dansyliert. Der Trockenrückstand des Dansylderivats wird in 1 ml Äthanol gelöst.

Alle genannten Chemikalien haben p.a. Reinheitsgrad.

Laufmittel

Als Laufmittel wurden verwendet: Cyclohexan—Essigsäureäthylester—Es-

sigsäure (60:40:2); (B) Chloroform—Methanol—Essigsäure (100:3:1); (C) Cyclohexan—Essigsäureäthylester (70:30). Alle genannten Laufmittel werden nach einmaligen Gebrauch verworfen.

Sprühmittel

Als Sprühmittel wurde verwendet Isopropanol—Triäthanolamin (80:20).

METHODE

(a) Extraktion

1 ml Serum wird in einem Schütteltrichter mit 1 ml wasser, 0.2 ml Acetatpuffer von pH 4.3 und 5 µl der Mafenid-Standardlösung versetzt, mit 1 × 10 ml und 1 × 5 ml Chloroform—Isopropanol (100:8) extrahiert, die organische Phase durch eine Faltenfilter (Selecta Nr. 595-1/2-Ø 9 cm) filtriert, das Lösungsmittel im Luftstrom abgedampft, die Gefässwand mit 1 ml Aceton nachgespült und das Aceton ebenfalls abgedampft.

(b_1) Alkalische Hydrolyse

Der Extraktionsrückstand wird in 1 ml 5 *N* NaOH aufgenommen und im offenem Röhrchen während 2 h auf 100° erhitzt. Die gut abgekühlte Lösung wird mit einem durch Titration gegen Phenolphthalein im Blindversuch ermittelten Volumen HCl 1:1 neutralisiert (0.84 ml).

(b_2) Saure Hydrolyse

Diese wurde durchgeführt mit 0.5 ml 50% (v/v) H_2SO_4, wobei 6 h auf 100° erhitzt wird. Die gut abgekühlte Lösung wird mit einem in Blindversuch ermittelten Volumen 5 *N* NaOH neutralisiert (1.85 ml).

(c) Dansylierung

Da unter den hier vorliegenden Reaktionsbedingungen, die durch relativ hohe Salzkonzentrationen gekennzeichnet sind, die Dansylierung mit $NaHCO_3$-Puffer unvollständig verlief und sich mit Na_2CO_3-Puffer wegen des Vorhandenseins der schwach aciden Sulfonamidgruppe bereits ein Na-Salz bildet, war es notwendig, ein $NaHCO_3$—Na_2CO_3-Puffer einzusetzen: Die neutralisierten Lösungen werden mit 2 ml $NaHCO_3$—Na_2CO_3-Puffer von pH 9.5 und 2 ml Dansylchloridlösung versetzt und 30 min auf 45° erwärmt. Nach dem Abkühlen wird mit 1 × 2 ml und 1 × 1 ml Chloroform extrahiert, die untere Phase mittels einer Pipette abgehoben und durch ein Faltenfilter (Selecta Nr. 595-1/2-Ø 5.5 cm) in ein spitzes Röhrchen filtriert. Das Lösungsmittel wird im Luftstrom abgeblasen.

(d) DC-Trennung

Die Dansylderivate werden in 50 µl Chloroform gelöst und in Anteilen zu 5 µl unter Zwischentrocknung auf eine DC-Platte (10 × 20 cm, Kieselgel 60, Merck) unter Einhaltung von 2 cm Abstand von den Plattenrändern aufgetragen. Im Abstand von 5 cm zur Probe werden auf dieselbe Stelle je 5 µl Glipizid- und Mafenid-Standardlösung mit je 250 ng nicht dansylierter Substanz aufgetragen. Die Trennung erfolgt durch zweidimensionale DC im Dunkeln zunächst mit dem Laufmittel A bis zum Plattenrand. Zum Trocknen wird die Platte 15 min im Dunkeln stehen gelassen, anschliessend wird mit dem Lauf-

mittel B ebenfalls bis zum Plattenrand entwickelt, getrocknet und kräftig mit Isopropanol—Triäthanolamin bis zur beginnenden Transparenz besprüht. Zum Nachtrocknen wird die Platte 30 min im Dunkeln gelagert.

(e) Fluoreszenzmessung

Anregung bei 366 nm, Emission bei 510 nm, ausgeleuchtete Fläche: 4 mm × 6 mm, Monochromatorblende ganz geöffnet, maximale Verstärkung (3000 ×). Zur Erhöhung der Empfindlichkeit und zur Unterdrückung von 0-Linienschwankungen wird eine zweite Kieselgelplatte unterlegt. Probe, Glipizid-, äusserer Mafenid- und innerer Mafenidstandard werden unter der UV-Lampe markiert und durch Scannen in der zweiten Eluierungsrichtung aufgenommen. Die Peakhöhen sind der Reihe nach h_x, h_{st}, H_m und h_m. m_{st} sei die aufgetragene Menge an Glipizidstandard (250 ng).

(f) Berechnung

Die Glipizidmenge m_x/ml Serum wird wie folgt berechnet

$$m_x = \frac{h_x \cdot H_m}{h_{st} \cdot h_m} \cdot m_{st}$$

ERGEBNISSE UND DISKUSSION

Wegen der starken Konzentrierung des Serumextraktes treten bei eindimensionaler DC störende Überlagerungen auf. Eine vollständige Trennung wird durch zweidimensionale DC erreicht*. Eine zusätzliche Reinigung der Serumextrakte durch eine extraktive Methylierung [13—15] scheidet zumindest bei der Glipizid-Bestimmung aus, da Pyrazin mit Methyljodid ein quartäres Ammoniumsalz bildet, welches sich nicht extrahieren lässt.

Überlagerungen im Chromatogramm können auch bei benachbarten, vollständig getrennten Substanzen auftreten, wenn zur Erzielung einer grossen Empfindlichkeit die DC-Platte mit einem breiten Lichtbündel ausgewertet wird. Solche, durch eine unvollständige optische Auflösung hervorgerufene Überlagerungen werden vermieden, wenn störende Substanzen unter der UV-Lampe abgekratzt werden. Voraussetzung ist, dass fehlende Streulichtanteile durch eine unterlegte zweite DC-Platte wieder ergänzt werden können.

Die mit den Laufmitteln A, B und C erhalten R_F-Werte der drei Glipizid-Spaltprodulte und des Mafenid-Standards sind in Tabelle I aufgeführt, ebenso die in Frage kommenden mengenmässig bedeutsamsten Metabolite, das 4-*trans*-hydroxy-cyclohexyl- und das 3-*cis*-hydroxy-cyclohexyl-Derivat, die bei der alkalischen Spaltung in die entsprechenden Hydroxyderivate des N-(4-Aminoäthylbenzolsulfonyl)-N′-cyclohexyl-harnstoffs übergehen.

Zur Überprüfung der Linearität und der Streuung wurden Messungen im Bereich von 10—500 ng pro Fleck durchgeführt, wobei jede Probe für sich hydrolisiert und dansyliert wurde. Die Auftragung Peakhöhe—Menge ergibt eine lineare Beziehung. Der Variationskoeffizient der Methode ab der Extraktion beträgt ± 4.3% (9 Bestimmungen im Bereich von 25—500 ng), die Nachweisgrenze 1 ng/ml Serum und die Wiederfindung 83 ± 3.2% (5 Proben zu je

*Inzwischen konnte auf eindimensionale DC übergegangen werden, wobei Kieselgelplatten mit Konzentrierungszone (Merck, Darmstadt, B.R.D.) und Äther (max. 0.2% Wasser!) oder Äther—Essigsäureäthylester—Cyclohexan (50:25:25) als Laufmittel eingesetzt wurden.

TABELLE I

R_F-WERTE VON DANSYLDERIVATEN DER SPALTPRODUKTE VON GLIPIZID UND SEINER METABOLITE

Amin	Laufmittel		
	A	B*	C
N-(4-Aminoäthylbenzolsulfonyl)-N'-(4-trans-hydroxycyclohexyl)-harnstoff	0.03	0.07	
N-(4-Aminoäthylbenzolsulfonyl)-N'-(3-*cis*-hydroxycyclohexyl)-harnstoff	0.04	0.09	
4-Aminomethylbenzolsulfonamid (Standard)	0.18	0.22	
4-Aminoäthylbenzolsulfonamid	0.19	0.29	0.01
N-(4-Aminoäthylbenzolsulfonyl)-N'-cyclohexyl-harnstoff	0.36	0.40	0.02
Ammoniak	0.43	0.38	0.19
Cyclohexylamin	0.69	0.83	0.49
Dansylchlorid	0.78	0.89	0.63

*Die R_F-Werte gelten bei zweidimensionaler DC, wenn zuvor mit dem Laufmittel A entwikkelt wurde, oder bei eindimensionaler DC mit erhöhten Methanolanteil des Laufmittels (Chloroform—Methanol—Essigsäure, 100:5:1).

250 ng Glipizid pro ml Serum).

Die vorliegende Methode eignet sich nicht nur für Routinebestimmungen, sondern auch für pharmakokinetische Untersuchungen, die bereits nach der radio-tracer Methode mit C^{14}-markiertes Glipizid durchgeführt wurden [16, 17].

DANK

Ich danke Herrn Prof. Dr. H. Grunicke, Vorstand des Instituts für Medizinische Chemie, für die Förderung der Arbeit und Herrn Prof. Dr. G. Wick, Vorstand des Instituts für Allg. und Exp. Pathologie, für die Bereitstellung des Chromatogramm-Spektralphotometers.

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Vol. 146, No. 3

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

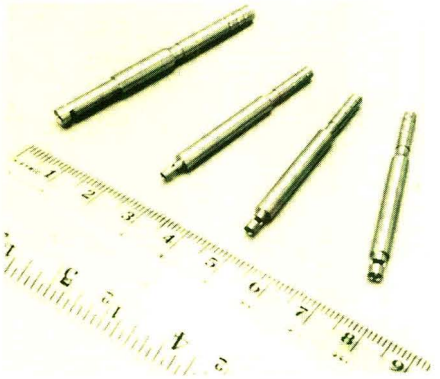
NEWS SECTION

APPARATUS

N-1154

GEL PUNCHES FOR SAMPLE WELLS

Now available from Bio-Rad Laboratories is a range of gel punches to provide uniform circular holes for sample wells. To prevent accidental suctioning of the gel surrounding the sample well, the punch has an outer tube for cutting and a sliding inner tube for suction. The suction is broken by air passing between the tubes. Sample well volumes in 1.5 mm thick gels are nominally 5, 8, 10 and 15 μl for the 2, 2.5, 3 and 4 mm diameter punches, respectively.



N-1130

ELECTROPHORESIS BROCHURE

Now available from Corning Medical is their 12-page illustrated brochure describing their agarose electrophoresis systems and procedures for protein separations. Equipment detailed includes Corning's cassette cell and power supply, incubator/oven, sample dispenser, film cutter, agarose gels and reagents. A chart presents sample requirements, gels and stains or substrates, wavelength for measurement and time required for tests such as serum proteins, lipoproteins, immunoelectrophoresis, etc.

N-1167

POWER SUPPLY FOR IMMUNOELECTROPHORESIS

Bio-Rad's Model 1450 power supply for immunoelectrophoresis and other electrophoretic techniques delivers constant voltage from 1–500 V at up to 200 mA and features a voltage probe to measure the actual potential across the gel in V/cm. Two units can be coupled in series to provide 1000 V d.c. or 400 mA maximum output. The unit has dual output jacks, polarity reversing switches, and a calibrated meter which reads current, voltage or cell voltage in V/cm.

N-1204

PACKARD RADIOCHROMATOGRAM SCANNER

Radiochromatography instruments are described in a new brochure published by Packard. The brochure gives information on radiochromatography instruments for paper, thin-layer, and gas chromatography. The radiochromatogram scanners Model 7220 and 7221 are subject to a minute description. These instruments are designed to detect radioactively marked compounds that are separated by thin-layer or paper chromatography. For the detection of compounds separated by gas chromatography Packard manufactures the Model 894 Proportional Counter. For off-line detection the Model 852 Gas Fraction Collector is described. It is designed to allow subsequent liquid scintillation counting.

N-1202

COMPUTING DENSITOMETER

The Gelman DCD-16 digital computing densitometer is available with or without the UV fluorometric mode. This option gives a greater flexibility of choice to laboratories performing low to moderate volumes of electrophoresis tests. The instrument has direct digital readout of relative percentage, has an automatic background correction, and manual or automatic peak selection. The grating monochromator allows wavelength selection within the range from 475 to 700 nm. Both micro and macro separations can be scanned, including the use of such media as cellulose acetate, agarose, or polyacrylamide gels. The carriage can be positioned either continuously or in steps for 8-track electrophoresis. The scan time is 15, 30 or 45 sec, with a maximum scan length of 10 × 6 cm.

For further information concerning any of the news items, apply to the publisher, using the reply cards provided, quoting the reference number printed at the beginning of the item.

N-1219

ELECTROPHORESIS POWER SUPPLIES

Three new regulated d.c. power supplies available from Isolab are designed for doing all types of electrophoresis requiring moderate voltage and minimum power to avoid denaturation of heat sensitive material. Two models provide constant voltage, current or power for the simultaneous operation of two electrophoresis units. The output voltage is continuously variable from 0 to 300 V d.c., the current from 0 to 200 mA, and the power from 0 to 60 W. The third model, a low-voltage, high-current d.c. power supply, is suited for fast electrophoretic destaining of polyacrylamide gels. The maximum output is 50 V d.c. at 1 A.

N-1171

AMINO ACID ANALYZER

Described in Durrum Dialogue No 4 1977 is the new Durrum Model D-400 analyzer which is a completely automatic system for the separation and measurement of amino acids in protein hydrolyzates and biological fluids. Up to 24 samples can be injected and analyzed by single-column ion-exchange chromatography, to a sensitivity of 0.5 nmole for each component. An integral ammonia filter continuously removes contaminating amines from buffers. Linear absorbance of ninhydrin-positive components is displayed on a two-pen recorder. Chromatographic procedures are controlled by a 15-channel programmer which can select six buffers and two column temperatures plus control accessories including an optional calculating integrator.

N-1200

CORNING 720 FLUORIMETER/ DENSITOMETER

Corning Medical has developed a semi-automatic fluorimeter/densitometer for the quantitative determination of iso-enzymes separated by electrophoresis. With the new microprocessor-controlled instrument, samples can be determined in 12 sec. The result is produced as a graph on an information card for easy storage. There is an accompanying viewer for study of the fluorimetric electrophoresis films before they are scanned. The instrument is capable of scanning all types of flat, transparent electrophoresis plates. The built-in microprocessor takes care of the automatic zero and the automatic readout of the instrument, and gives a warning signal in case of malfunction. The temperature of the electrophoresis plate is held constant within 1°, in order to guarantee a constant fluorescence excitation for the total plate. Every instrument is delivered with a non-biological testing pattern that can be used to check the instrument's functions.

CHEMICALS

N-1215

NEW ELECTROPHORESIS PRODUCTS

Pierce Chemical Company has expanded its offerings for polyacrylamide gel electrophoresis. In addition to acrylamide, methylene bis-acrylamide, TEMED, ammonium persulfate and sodium dodecyl sulfate, 50 other products designed for use in electrophoresis are described in the February Pierce newsletter, Previews. Also new is a protein molecular weight calibration kit for SDS–polyacrylamide gel electrophoresis, called Page Ranger™.

PROCEDURES

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NEW BOOKS

Solid phase methods in protein sequence analysis (Proc. 2nd Int. Conf., Montpellier, September 21–23, 1977), edited by A. Previero and M.-A. Coletti-Previero, North-Holland, Amsterdam, New York, Oxford, 1977, XVIII + 298 pp., price Dfl. 87.00, US$ 37.95, ISBN 0-7204-0654-4.

Electrofocusing and isotachophoresis (Proc. Int. Symp., Hamburg, August 2–4, 1976), edited by B.J. Radola and D. Graesslin, Walter de Gruyter, Berlin, New York, 1977, XVI + 608 pp., price DM 150.00, ISBN 3-11-007026-X.

Preparation and characterization of mammalian plasma membranes (Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 7, Part I, edited by T.S. Work and E. Work), by W.H. Evans, North-Holland, Amsterdam, New York, Oxford, 1978, II + 266 pp., price Dfl. 60.00, US$ 26.00, ISBN 0-7204-4222-2.

Recent developments in mass spectrometry in biochemistry and medicine, Vol. 1 (Proc. 4th Int. Symp., Riva del Garda, June 1977), edited by A. Frigerio, Plenum, New York, London, 1978, XII + 658 pp., ISBN 0-306-31138-0.

Separation methods for drugs and related organic compounds, by G. Schill, Apotekarsocieteten, Stockholm, 1978, VIII + 182 pp.

CALENDAR OF FORTHCOMING MEETINGS

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Barcelona, Spain

International Congress on Analytical Techniques in Environmental Chemistry

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Expoquimia, av. M.ª Cristina, Palace n.º 1, Barcelona-4, Spain

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Biochemical Separation Methods (Uppsala Separation School)

Contact:
Eva Linder, Institute of Biochemistry, University of Uppsala, Box 576, S-75123 Uppsala, Sweden

March 5–8, 1979
Zurich, Switzerland

3rd European Symposium on Vitamin B_{12} and Intrinsic Factor

Contact:
Dr. B. Zagalak, Chemisches Laboratorium, KISPI, Universität Zürich, Steinwiesstrasse 75, CH-8032 Zürich, Switzerland

April 18–20, 1979
Carlsbad, Czechoslovakia

2nd Danube Symposium on Progress in Chromatography

Contact:
Dr. Karel Macek, Institute of Physiology CSAV, Budejovicka 1083, Prague 4, Czechoslovakia (Further details published in Vol. 160, No. 1)

May 7–10, 1979
Boston, Mass., U.S.A.

4th International Symposium on Column Liquid Chromatography

Contact:
Professor Barry L. Karger, Chairman, Organizing Committee, 4th International Meeting on Column Liquid Chromatography, Northeastern University, Institute of Chemical Analysis, Boston, Mass. 02115, U.S.A. (Further details published in Vol. 154, No. 2)

June 19–20, 1979
Venice, Italy

10th International Symposium on Chromatography and Electrophoresis

Contact:
Dr. A. Frigerio, Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan, Italy

June 21–22, 1979
Venice, Italy

6th International Symposium on Mass Spectrometry in Biochemistry and Medicine

Contact:
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September 24–27, 1979
Lausanne, Switzerland

Chromatography '79. 14th International Symposium on Advances in Chromatography

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2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
3 H. C. S. Wood and R. Wrigglesworth, in S. Coffey (Editor), *Rodd's Chemistry of Carbon Compounds, Vol. IV, Heterocyclic Compounds*, Part B, Elsevier, Amsterdam, Oxford. New York, 2nd ed., 1977, Ch. 11, p. 201.
4 E. C. Horning, J.-P. Thenot and M. G. Horning, in A. P. De Leenheer and R. R. Roncucci (Editors), *Proc. 1st Int. Symp. Quantitative Mass Spectrometry in Life Sciences, Ghent, June 16–18, 1976*, Elsevier, Amsterdam, Oxford, New York, 1977, p. 1.

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